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APPENDIX B

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Platelet Adhesion

INTRODUCTION

For the purposes of this review, platelet adhesion is defined as platelet adherence to cells, tissues, surfaces, and particles, but platelet-to-platelet adhesion, that is, platelet aggregation, is excluded.

It seems appropriate to begin a discussion of platelet adhesion by emphasizing that platelets do not adhere to the intact, undamaged endothelial surface of a normal blood vessel.(1–6) The reasons for this are not yet fully understood, but the inability of platelets to adhere to this surface is essential for the maintenance of a vascular tree through which blood can flow freely. Platelet adhesion, therefore, is generally the response to an abnormal state of a blood vessel, or to the presence of an abnormal surface.

Platelet adhesion plays a central role in the formation of hemostatic plugs and thrombi, particularly arterial thrombi, although venous thrombi may be initiated by a mass of aggregated platelets that accumulate on adherent platelets in a valve pocket of an injured vein. Platelets may also adhere to diseased vessel walls and to abnormal or diseased cardiac valves, but this aspect of platelet adhesion has received little attention since most experimental work has been done with normal vessels of comparatively young animals. The release of platelet-derived growth factor from platelets that adhere to an injured vessel wall has been implicated in the initiation of the smooth muscle cell proliferation that characterizes atherosclerotic lesions. Platelet adhesion appears to have a role in some aspects of wound healing. Maintenance of the integrity of the endothelial lining of blood vessels is also one of the major roles of blood platelets as is well illustrated by the easy bruising that is characteristic of thrombocytopenia.

Platelets adhere readily to the subendothelium, to the injured neointima that forms when a vessel has been repeatedly damaged, to the deeper constituents of a vessel wall when these are exposed, to monocytes, macrophages and some other cells, to polymeriz-

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ing fibrin, to a wide variety of particulate material, and to artificial surfaces. All these aspects of platelet adhesion except adhesion to artificial surfaces will be discussed in turn.

ADHESION IN HEMOSTATIC PLUGS

When a small vessel is severed, or a larger vessel is punctured, platelets accumulate rapidly at the site and form a hemostatic plug composed of aggregated platelets.(7–14) Within a few minutes, fibrin forms around the plug and stabilizes it. The platelets in contact with the cut edges of the tissue have been shown to be adherent to collagen fibers with gaps in their membranes that are adjacent to the collagen fibers.(10) Some of these platelets are swollen, and have lost their internal structures. The platelets at the periphery of the plug become adherent to polymerizing fibrin (15) formed under the influence of thrombin that is likely generated upon activation of the extrinsic coagulation pathway by tissue thromboplastin from the damaged cells of the vessel wall (see Fig. 1, p. 224).

ADHESION TO THE SUBENDOTHELIUM IN VIVO

The adhesion of platelets to the injured site is one of the first events that follows removal of the endothelial lining of a normal blood vessel that has not been injured previously.(5, 16–22) These observations have been made with rabbits, rats, monkeys, pigs, and dogs. The subendothelium becomes completely covered by a layer of adherent platelets within minutes, probably as quickly as platelets can be brought into contact with the surface by the hemodynamic forces of flowing blood. If blood flow is rapid and laminar, thrombi do not form on this initial layer of adherent platelets. In regions where flow is disturbed and vortices and eddies can form, however, additional platelets adhere to the platelets on the wall and to each other, forming an aggregate, or platelet thrombus.

The reactions that are triggered in the platelets as a result of their adhesion to the constituents of the subendothelium play a major part in the formation of a thrombus where blood flow is disturbed. The contents of the alpha and amine storage (dense) granules are released and the ADP and serotonin from the latter act synergistically to cause platelet aggregation. Phospholipases are activated, freeing arachidonate, which is converted to products that cause aggregation and may affect adhesion. The surface of the platelets is altered so that phospholipid becomes available and allows binding of coagulation factors to the platelets to accelerate the intrinsic coagulation pathway. In addition, binding sites are exposed for proteins that are involved in platelet adhesion and platelet aggregation. These reactions will be discussed in detail in subsequent sections of this article. Activation of the coagulation pathways at the injury site, and on the surface of the adherent and aggregating platelets, causes the local formation of thrombin, which influences thrombus formation and stabilization in several ways: thrombin causes further platelet aggregation, further release of platelet granule contents, further formation of the aggregating agents that arise from arachidonate, and the same changes in the surface of platelets that result from their adherence to the injury site; thrombin causes fibrin to form around and among the platelets, stabilizing the aggregate since platelets adhere to polymerizing fibrin; thrombin may also limit thrombus formation by activating protein C and stimulating PGI_2 formation by intact endothelium adjacent to the injury site. The eventual fate of a thrombus varies from dissolution, as the platelets deaggregate and fibrin is lysed by plasmin, to

organization and incorporation of a persistent thrombus into the wall, thus contributing to vessel wall thickening.

Detailed morphologic studies by electron microscopy have revealed several stages in the process of platelet adhesion to the subendothelium of previously uninjured vessels. The platelets first contact the surface, then become adherent as further interactions between their plasma membrane and the surface occur, and finally spread out on the surface, forming a layer of flattened platelets. Electron micrographs taken at high magnifications reveal periodic bridges between adherent platelets and the dense bands of collagen fibrils.(13, 23) Platelets that have merely contacted the surface and have not yet changed their shape to an appreciable extent, as well as some platelets in the initial stages of adhering, may be removed by the force of blood flow, but platelets that have spread on the surface do not appear to detach readily. Most of them remain on the surface for several days. The observations supporting this conclusion have been obtained in vivo with experimental animals, mainly rabbits and rats, into which ^{51}Cr-labeled platelets have been injected before the removal of the endothelium from the aorta with a balloon catheter. Examination of the radioactivity of the aorta of animals killed at time intervals ranging from 10 minutes to 7 days has revealed that most of the platelets that adhere initially remain on the surface for several days.(5) If the endothelium is removed before injection of labeled platelets, much less ^{51}Cr becomes associated with the wall because the labeled platelets do not replace the unlabeled platelets that adhere immediately following the exposure of the subendothelium. If the animals (rabbits or rats) are given heparin intravenously immediately before the aortae are perfusion-fixed in situ, fibrin is not seen on the subendothelium in association with the adherent platelets.(2, 5, 24, 25) Investigators who describe fibrin in association with a vessel subjected to injury with a balloon catheter either have not taken precautions to prevent fibrin formation during removal of the blood and fixation of the vessel, or have injured deeper tissues in the vessel wall as well as removing the endothelium. This latter assessment of their finding arises from the observations that injury of smooth muscle cells causes thrombi to form with a significant fibrin component as well as with aggregated platelets. A further indication that fibrin is not involved in the adherence of platelets to the subendothelium of a previously uninjured vessel is the observation that the administration of heparin before, and for 24 hours, after removal of the endothelium with a balloon catheter does not significantly influence the number of platelets that are associated with the surface during this time.(5)

The initial layer of platelets that forms on the subendothelium of large vessels within the first few minutes of removal of the endothelium does not present a surface to the blood that attracts additional platelets in the way that the exposed subendothelium initially attracted platelets.(3, 5, 26) The reason for this is not clear, but is undoubtedly related to the surface characteristics of the platelets that cover the de-endothelialized area. Possibly because laminar blood flow rapidly removes the ADP and thromboxane A_2 that are lost from the adherent platelets, insufficient amounts of these aggregating agents can accumulate at the site to make the fibrinogen receptors on the surface of the circulating platelets available for platelet aggregation on the adherent platelets. Adhesion on the subendothelium reaches a maximum in vivo in rabbits by 10 minutes, although a large proportion of the platelets are likely adherent well before this time.(5) Evidently adhesion stops when the reactive areas of the subendothelium become completely covered with platelets.

To answer the question of whether the subendothelium becomes less attractive to platelets with time even if it is not covered by platelets, platelet adhesion was inhibited by administration of dipyridamole or PGI_2 before removal of the endothelium.(27) Treatment

with dipyridamole was continued for several hours afterwards. Inhibition of the initial platelet adhesion by dipyridamole over a 4 hour period, or PGI_2 over 10 minutes, did not inhibit platelet accumulation when the treatment was discontinued, but treatment of the rabbits with dipyridamole for 8 hours did decrease the number of platelets that accumulated after the concentration of dipyridamole in plasma fell to ineffective concentrations. It is apparent that even without complete coverage with spread platelets, the subendothelium eventually loses its reactivity to circulating platelets, although this change in its properties requires more than 4 hours.(27) In experiments in which no treatments are given, platelets are gradually lost from the subendothelium and, by 48 hours, the subendothelium is poorly reactive to circulating platelets.(3, 5) If ^{51}Cr-labeled platelets are reinjected at this time, few of them adhere to the subendothelium.(5, 27) The changes in the subendothelium that cause it to become less and less attractive to platelets are not understood. PGI_2 production by the vessel wall can be ruled out because treatment of the rabbits with aspirin in doses sufficient to block PGI_2 formation completely does not influence the number of platelets adherent to the subendothelium at any time after the injury.(6) It is unlikely that a coating with a plasma protein (such as albumin, which tends to passivate artificial surfaces) is responsible for the gradual loss of reactivity to platelets, because much more rapid adsorption of circulating proteins would be expected. Possibly enzymatic reactions occur to change the adhesive properties of the components of the subendothelium to which platelets adhere. Enzymes that might be present include collagenase and elastase from platelets or leukocytes (28–30) and the Ca^{2+}-activated protease from disrupted platelets.(31)

Electron microscopy of platelets adherent to the subendothelium reveals that most of them have lost many of their storage granules.(32) Baumgartner and co-workers (33) have shown that platelets adhere to collagen fibers, basement membrane, and the microfibrils around elastin in the subendothelium, but not to elastic fibers, proteoglycans, or amorphous material. In their electron micrographs, only platelets adherent to collagen seem to have released their granule contents, although this may be open to reinterpretation, since Fauvel and colleagues (34) have shown that in the presence of von Willebrand factor, platelets that adhere to microfibrillar material isolated from vessel walls also release their granule contents. Huang and Benditt (35) have also shown that, although platelets adhere to the glomerular basement membrane and spread on it, they do not release their granule contents. Other investigators, however, have observed release and aggregation when platelets adhere to glomerular basement membrane.(36, 37)

Many detailed studies have been done in vitro of platelet adhesion to the subendothelium or to its constituents, particularly to collagen. These will be discussed in later sections.

ADHESION TO DAMAGED NEOINTIMA IN VIVO

The response of the blood to reinjury, that is, injury of the neointima that forms after de-endothelialization, differs in a number of ways from the response to exposed subendothelium. In 1973, Stemerman observed that thrombi composed of both platelets and fibrin formed on the injured neointima of rabbit aortae.(18) These findings have been confirmed and extended.(38, 39) The neointima that forms 4 to 7 days after removal of

the endothelium is largely composed of smooth muscle cells, so that a second injury with a balloon catheter after this time damages these cells. It seems likely that tissue thromboplastin becomes available for the extrinsic pathway of coagulation and is responsible for the extensive formation of fibrin under these circumstances. The number of platelets that accumulate is similar to the number that adhere to the subendothelium after an initial injury (38); some of them are trapped in the fibrin meshwork, whereas others appear to be adherent as a single layer of platelets to connective tissue and noncellular, amorphous material in the injured vessel wall. (The term *platelet accumulation* is used to indicate that some of the platelets associated with the wall may be aggregated platelets trapped in fibrin rather than platelets actually adherent to the damaged wall.) The thrombi tend to be oriented in the direction of blood flow, and this is particularly evident at vessel orifices where they are distributed in a pattern that curves toward the opening of the branch. In some areas leukocytes can also be observed on the surface, many of them in a zone distal to the orifices of small vessels. Some of the leukocytes are adherent to the platelets that are attached to the injured surface. As would be expected, administration of heparin inhibits platelet accumulation on the damaged neointima by about 50 percent and fewer platelet-fibrin thrombi are observed.(38) It seems likely that in the presence of heparin, only the adhesion of platelets to connective tissue occurs since this process is not dependent on blood coagulation. Heparin evidently inhibits platelet accumulation that is mediated by fibrin. These observations indicate that fibrin formation under the influence of thrombin, and platelet adhesion to polymerizing fibrin, may play a much larger part in the initiation and growth of thrombi on repeatedly injured vessels than has been apparent from studies of thrombosis on normal vessels subjected to removal of the endothelium. Indeed, at sites where hemodynamic forces would be expected to cause repeated vessel injury, fibrin has been observed in contact with the wall, with platelets and other formed elements of the blood adherent to it, in sections taken from otherwise normal experimental animals or from humans at autopsy (see Fig. 2, p. 241).(40) Injuries that damage tissue deep in the vessel wall cause the formation of thrombi that have fibrin at their point of attachment.(41) If some thrombi are formed largely under the influence of thrombin, it is apparent that anticoagulants would be more effective than drugs that inhibit platelet aggregation, in limiting thrombosis, and that the combination of an anticoagulant with a drug that inhibits platelet adhesion as well as aggregation would be most effective, providing the risk of hemorrhage is not increased unduly.

The accumulation of platelets on the injured neointima is at its maximum within 60 minutes after passage of the balloon catheter.(38) However, the loss of radioactive platelets that have become associated with the injured surface of the neointima occurs somewhat more rapidly than their loss from the subendothelium, and by 24 hours, only about one third of the initial number are present.(38) By this time, very few thrombi can be found on the surface and it seems likely that fibrinolysis has a role in their removal.

^{51}Cr-labeled platelets were injected into rabbits at different times after the neointima was injured to examine the ability of the damaged surface to attract fresh platelets and continue to activate coagulation. Platelet accumulation was greatly reduced one hour after injury, and by 4 days, only about 10 percent of the number of platelets accumulated, if the number of platelets that accumulated immediately after injury was taken as 100 percent.(38) Piepgras and colleagues have also observed that endarterectomy of the carotid arteries of cats exposes a surface that becomes nonthrombogenic in about 6 hours if the initial deposition of thrombi on the surface is inhibited.(42)

PLATELET ADHESION TO DISEASED ARTERIES

Atherosclerotic lesions frequently have thrombi associated with their surface,(43–45) and the formation of these thrombi must involve platelet adhesion, probably occurring after loss of the endothelium covering the plaque. Thrombi in coronary arteries are almost always associated with breaks in the vessel wall at atherosclerotic plaques.(46–51) Platelet aggregates have frequently been described in direct contact with materials in atherosclerotic lesions,(48, 52, 53) although the components of the lesions vary considerably. Lipid-rich plaques that contain cholesterol crystals are likely to promote platelet adhesion, since platelets have been shown to interact with cholesterol crystals.(54) In monkeys fed hypercholesterolemic diets, platelets have been observed adherent to macrophages in lesions on the surface of large arteries.(55)

In general, our understanding of platelet adhesion to the contents of atherosclerotic plaques is limited. There have been very few experimental studies and most of the observations come from examination of post mortem material.

PLATELET ADHESION IN VEINS

Venous thrombi are similar to a blood clot in that they consist mainly of red blood cells trapped in a fibrin mesh. Usually they are not adherent to the vessel wall except, under some circumstances, at the point of initiation where a white head of aggregated platelets is demonstrable.(56, 57) This may occur in a valve pocket. Many venous thrombi are stasis thrombi that form because blood coagulation occurs. Stasis results in adhesion of polymorphonuclear leukocytes to the endothelium and their migration into the wall. However, these events do not necessarily cause obvious endothelial cell damage or loss, and platelet adherence does not occur when blood flow is restored.(58) Under circumstances in which there is extensive tissue damage, however, such as that resulting from hip surgery, injury to the vessel wall does play a major part in the initiation of venous thrombi.(59) Extensive adhesion of leukocytes to the walls of veins has been reported following surgery (60, 61); endothelial cells separate from each other and are lost at some sites. This can promote platelet adhesion and thrombus formation on the injured surface.

MAINTENANCE OF THE INTEGRITY OF THE ENDOTHELIUM

Platelets are necessary to maintain normal vessel wall integrity.(62, 63) It is well known that in severe thrombocytopenia, the vascular walls become more permeable,(64) and easy bruising occurs as red cells escape from capillaries.(62) The early estimate that a turnover of 8000 to 12,000 platelets/μL/day was a critical number, below which spontaneous bleeding is likely to occur readily,(65) has recently been confirmed by Hanson and co-workers,(66) who have calculated that 10 to 15 percent of the circulating platelets in normal subjects are randomly consumed in maintaining vascular integrity (7300 platelets/μL/day). They point out that this platelet requirement produces predictably shortened platelet survival in the patients with aplastic thrombocytopenia whom they studied, and may contribute to shortened platelet survival in other thrombocytopenic states. It should be pointed out that shortened platelet survival is demonstrable in such patients only if the

studies are done at a low platelet count.(65) In order to maintain the endothelial lining it is apparent that platelets must adhere to the vessel wall, but practically nothing is known about this process. Electron micrographs illustrate that platelets interact with the vessel wall if there are gaps between endothelial cells.(67–70) The early suggestion that the endothelial cells actually engulf platelets (62) is no longer accepted.

CONDITIONS THAT RESULT IN PLATELET ADHESION TO CULTURED ENDOTHELIAL CELL MONOLAYERS

Although unstimulated platelets do not adhere to normal endothelium, nor to damaged endothelium in vivo,(26, 71, 72) they apparently can adhere to damaged or altered endothelium in culture.(73–75) Booyse and colleagues damaged endothelium cells in culture in various ways and observed that platelets did not interact with the damaged cells per se, but rather with an extracellular matrix of microfilaments produced by the endothelial cells.(73) In the experiments of Curwen and coworkers,(74, 75) platelets were shown to adhere to virally transformed endothelial cells in culture. Although exogenous PGI_2 had a partial inhibitory effect on platelet adhesion to these endothelial cells, it did not depress platelet adhesion to the basal level observed with normal endothelial cells in culture. The investigators concluded that exposure to the virus altered the property of endothelial cells that prevents platelet adhesion. They also concluded that generation of PGI_2 by the normal endothelium is not the key factor that prevents platelet adherence to the intact vessel wall.

Other investigators have reached the opposite conclusion, although their results are open to alternative explanations. Czervionke and colleagues (76, 77) reported that ^{51}Cr-labeled platelets that were exposed to thrombin adhered as aggregates to cultured endothelial cells, providing PGI_2 production by these cells was blocked by aspirin. This thrombin-induced adhesion was inhibited by the addition of PGI_2 (5 to 25 n*M*). Curwen and colleagues (74) have pointed out, however, that platelet adherence cannot be readily distinguished from platelet aggregation in such a test system, and PGI_2 may have been preventing platelet aggregation or causing deaggregation rather than inhibiting platelet adhesion. An additional explanation for the observations of Czervionke and associates (76, 77) may be that thrombin also causes the release of platelet fibrinogen and converts it to fibrin, which has been shown to bind to both platelets (15) and endothelial cells,(78, 79) and would form an adhesive bridge between them. Yet another possibility is that thrombin damaged the endothelium, as shown by Lough and Moore,(79) resulting in a surface to which platelets could adhere.

INTERACTION OF PLATELETS WITH CONSTITUENTS OF THE VESSEL WALL

Platelets have been shown to adhere to collagen, basement membrane, microfibrils, damaged smooth muscle cells, and components of atherosclerotic plaques. Adherence to damaged endothelial cells and other injured cells such as fibroblasts is less well established. By far the greatest number of studies have been devoted to investigating the adhesion of platelets to collagen, with in vitro studies of the interactions of platelets with collagen outnumbering the in vivo studies. Much less is known about the reactions that

occur as a result of the adhesion of platelets to other constituents of the vessel wall. It seems likely that although platelets may initially contact only one constituent of the vessel wall, when they spread on the surface they may become adherent to several vessel wall constituents.

INTERACTION OF PLATELETS WITH COLLAGEN, AND RELEASE OF GRANULE CONTENTS

More than 20 years ago, Hughes,(80) Bounameaux,(81) and Roskam (82) observed that vessel wall injury exposed subendothelial constituents that initiated platelet aggregation. Kjaerheim and Hovig (83) demonstrated that platelets were adherent to collagen when mesenteric blood vessels were injured. Zucker and Borrelli (84) and Hovig (85) showed that connective tissue or tendons contained material that caused platelets to aggregate, and collagen was identified as a platelet aggregating agent. These observations were made shortly after Gaarder and coworkers (86) had shown that ADP is an aggregating agent and Hovig,(87) Spaet and Cintron,(88) and Spaet and Zucker (89) went on to demonstrate that ADP was released from the platelets when they were stimulated with collagen. As a result of the experiments of Haslam (90) with enzyme systems that converted the released ADP to compounds that did not cause platelet aggregation, the concept arose that collagen and other aggregating agents such as thrombin caused aggregation because they induced the release of ADP from platelets. Although we now know that released ADP is not solely responsible for platelet aggregation induced by collagen because the aggregating agents formed from arachidonate also play a part,(91–97) the release of the contents of platelet granules when platelets adhere to collagen is an important aspect of this interaction.

From electron micrographs of hemostatic plugs, thrombi, and platelets on the subendothelium, the degranulation of platelets adherent to collagen in vivo is well established.(10, 12, 32) In regions where blood flow conditions are such that the materials released from the granules of platelets adherent to an injury site can accumulate, released ADP and serotonin are thought to act synergistically to contribute to platelet aggregation on the adherent platelets. Released ATP is converted to ADP by enzymes in plasma and thus increases the amount of ADP that accumulates.

In addition to the aggregating agents released from the amine storage granules of platelets adherent to collagen, several proteins are released from the α-granules. Recently, some evidence has accumulated that release of α-granule contents occurs more readily than the release of the contents of the dense granules.(98–100) The materials in the α-granules include platelet factor 4, ß-thromboglobulin, fibrinogen, fibronectin, thrombospondin, von Willebrand Factor, albumin, Factor V, antiplasmin, α_1-antitrypsin, α_2-macroglobulin, the platelet-derived growth factor that is mitogenic for smooth muscle cells, cationic proteins that increase vessel wall permeability, bactericidal factor, chemotactic factor, proteoglycans, and other proteins.(101, 102) Some of these, notably von Willebrand Factor, thrombospondin, and fibronectin, may be bound to the membrane of platelets that have undergone the release reaction, and these proteins have been implicated in platelet adhesion, particularly to collagen and microfibrillar material.(34, 103–111) In contrast, fibrinogen is required for platelet aggregation and binds to platelets on which the complex of glycoproteins IIb and IIIa have formed a receptor as a result of exposure of the platelets to aggregating agents.(112–119) It should be pointed out, however, that seem-

ingly adequate concentrations of von Willebrand's Factor, fibrinogen, and fibronectin exist in plasma to take part in adhesion and aggregation, and unless local high concentrations at the point of release from platelets are required to enhance these processes, it is difficult to understand how release of these proteins from platelets could be crucial. Thrombospondin has been identified as the lectin-like material from platelets that binds to the surface of platelets that have released their granule contents, and interacts with fibrinogen.(108, 109) It may have a role in platelet aggregation, particularly aggregation that is caused by release-inducing agents, and is not readily reversible, and possibly has a role in platelet adhesion. No role has been suggested for released ß-thromboglobulin, but because this protein seems to be unique to platelets, its appearance in plasma is taken as an indication that platelets have released granule contents.(120) Platelet factor 4, also called *antiheparin factor* interferes with the inhibition of thrombin caused by the antithrombin III complex with heparin. How it functions in the absence of heparin is unclear. Abnormally high concentrations in plasma occur when platelets have undergone the release reaction, but since platelet factor 4 becomes associated with the endothelial surface and is freed by heparin,(120) its concentration in plasma in vivo is less useful as an indicator of the extent of release of platelet granule contents than is the concentration of ß-thromboglobulin. Immunofluorescent studies have shown that, at a site where platelets adhere to exposed subendothelium, platelet factor 4 penetrates the outer layers of the vessel wall.(121) The other proteins released from adherent platelets undoubtedly also enter the injured wall.

The platelet-derived growth factor released from platelets adherent to an injured vessel wall stimulates the migration and proliferation of smooth muscle cells in the intima, and thus contributes to vessel wall thickening and the development of atherosclerotic lesions.(122, 123) If experimental animals are kept thrombocytopenic during repeated vessel wall injury, the lesions do not develop, indicating that platelet-derived growth factor is essential for the stimulation of smooth muscle cell proliferation under these experimental conditions.(124, 125)

Factor V that is released from adherent platelets may become associated with their surface, although in this case also, seemingly adequate concentrations are present in plasma. Activated Factor V on the platelet surface serves as the receptor for Factor Xa, and thus takes part in the prothrombinase complex that catalyses the conversion of prothrombin to thrombin.(126, 127)

Platelets that adhere to collagen may also release the contents of some of their lysosomal granules. Legrand and colleagues have shown that when platelets are incubated with collagen they convert their proelastase to elastase, which appears in the medium surrounding the platelets.(29) When this enzyme enters the injured wall to which platelets are adherent, it may lyse elastin. The proteolytic enzyme in platelets that exerts a limited hydrolytic effect on proelastase was not characterized in their study.

Formation of Aggregating Agents from Arachidonate

In the 1970s, several groups of investigators showed that when platelets interact with release-inducing agents, including collagen, the platelets form short-lived aggregating agents that affect other platelets in the vicinity.(91–94) These active substances were identified as the prostaglandin endoperoxides, G_2 and H_2 (PGG_2, PGH_2), and their product, thromboxane A_2. Thromboxane A_2 has an in vitro half-life of approximately 30 seconds in plasma at 37°C, and the half-lives of PGG_2 and PGH_2 are similarly short.(128, 129) They are formed from platelet arachidonate that is freed from platelet phospholipids

under the influence of phospholipases. It is not known how the process of platelet adhesion to collagen or to other surfaces, activates phospholipase, nor is it clear whether phospholipase A_2 or phospholipase C followed by diglyceride lipase is primarily responsible for freeing arachidonate.(130–132) The phospholipids from which arachidonate is hydrolyzed are mainly phosphatidyl inositol and phosphatidyl choline. The reactions involved in the conversion of arachidonate to thromboxane A_2 have been thoroughly described by several groups of investigators.(128, 129, 133) The enzymes responsible for the reactions are cyclo-oxygenase, which converts arachidonate to PGG_2, and thromboxane synthetase, which converts PGH_2 to TXA_2.

Because cyclo-oxygenase is inhibited by nonsteroidal antiinflammatory drugs such as aspirin, indomethacin, ibuprofen, and many others, these drugs prevent the formation of the prostaglandin endoperoxides PGG_2 and PGH_2, and thromboxane A_2, and thus inhibit collagen-induced platelet aggregation. Most investigators have found, however, that these drugs do not inhibit platelet adhesion to collagen or to other subendothelial components,(94, 134–138) nor do they inhibit the release of granule contents from platelets that adhere to collagen.(97) The failure of aspirin to inhibit release from adherent platelets has been shown morphometrically by in vivo experiments by Weiss and colleagues,(135) who described degranulated platelets adherent to collagen in sections taken from animals given sufficient aspirin to inhibit thromboxane A_2 formation and prevent collagen-induced aggregation, tested in vitro in citrated platelet-rich plasma. Further evidence for the conclusion that the formation of active compounds from arachidonate is not required for platelet adhesion to collagen or the release of granule contents from the adherent platelets comes from the experiments of Kinlough-Rathbone and coworkers,(97) and Cazenave and colleagues.(137) In these studies, platelets doubly-labeled with ^{51}Cr (a cytoplasmic label) and ^{14}C-serotonin (a label for the amine storage granule contents, which is rapidly taken up and sequestered in these granules) were used to quantify platelet adhesion and the extent of release of ^{14}C-serotonin from the adherent platelets. Since ^{51}Cr is not lost from the cytoplasm when platelets adhere, because they do not lyse, this label indicates the number of adherent platelets. When ^{14}C-serotonin is released, the ratio of ^{51}Cr to ^{14}C increases in the adherent platelets, and the extent of release can be calculated. In vitro experiments showed that aspirin did not affect the number of platelets that adhered nor did it decrease the release of ^{14}C-serotonin from the platelets adherent to collagen or to the subendothelium.(97) Results obtained by Cazenave and colleagues (137) demonstrated that other nonsteroidal antiinflammatory drugs also did not lessen adhesion or decrease the release of granule contents from adherent platelets, with the exception of indomethacin and high concentrations of sulfinpyrazone, which had small inhibitory effects on platelet adhesion. All these experiments with doubly-labeled platelets were done under conditions in which platelet aggregates were not present on the surfaces so that only platelets that were directly adherent to collagen or the subendothelium were examined. In the experiments of Legrand and associates,(139) aspirin did not affect platelet adhesion to collagen, but it did diminish the release of serotonin from the platelets, although this effect of aspirin may have been on platelets that were not adherent to collagen. Other studies of the effects of nonsteroidal antiinflammatory drugs on platelet adhesion are discussed under *Inhibitors of Platelet Adhesion.*

It must be emphasized that the situation in an aggregometer cuvette when collagen is added to citrated platelet-rich plasma or to a suspension of platelets in artificial media is quite different from the situation in the adherence experiments described above. In an aggregometer cuvette, a relatively small proportion of the platelets actually adhere to

collagen. By far, the majority of them do not contact collagen and are induced to aggregate by the synergistic effect of thromboxane A_2 formed by the adherent platelets, and the ADP and serotonin released from them. Nonadherent platelets stimulated by thromboxane A_2 also release granule contents and form more thromboxane A_2, amplifying the reaction. When aggregation is due to a synergistic effect of ADP and thromboxane A_2, aggregation can be largely prevented either by inhibiting the action of cyclo-oxygenase with nonsteroidal antiinflammatory drugs, or by removing ADP rapidly with an enzyme system such as creatine phosphate–creatine phosphokinase, which converts ADP to ATP.(140) Since neither ADP nor TXA_2 is a strong agonist, release of the contents of the amine storage granules rarely exceeds 50 to 60 percent, even when maximum aggregation occurs in an aggregometer cuvette. In contrast, a strong agonist such as thrombin can cause more than 90 percent release. Less than maximal release in response to collagen in an aggregometer cuvette is in accord with the principle pointed out by Huang and Detwiler (141) that a combination of agonists that act synergistically does not cause a greater response than the response evoked by a high concentration of the stronger of the two agonists.

If a very large amount of collagen is added, so that nearly all the platelets can adhere to it, massive aggregation occurs and release of granule contents to the extent of more than 80 percent takes place.(97) This represents release from the platelets that are actually adherent to the collagen, rather than release caused by thromboxane A_2. If a large amount of collagen is added to platelets in which cyclo-oxygenase has been inhibited, in an aggregometer cuvette, a gradual increase in light transmission occurs as the platelets adhere to collagen and, although the platelets do not aggregate, release of granule contents can be demonstrated.(97)

Although there are contradictory reports in the literature,(142) the most convincing studies indicate that ADP is not required for the adherence of platelets to collagen or to the subendothelium.(143, 144) Enzyme systems, such as creatine phosphate–creatine phosphokinase, which rapidly converts released ADP to ATP (an inhibitor of ADP-induced platelet aggregation), do not inhibit the adhesion of platelets to collagen or the subendothelium.(143, 144) Such an enzyme system does inhibit platelet aggregation on the adherent platelets. It seems likely that in studies in which removal of ADP has been reported to inhibit platelet adhesion, both adhesion and aggregation were occurring. Conversely, the demonstration of enhancement of platelet adhesion by the addition of ADP undoubtedly represents ADP-induced aggregation on the adherent platelets. Inhibition of platelet adhesion to collagen by pretreatment of the platelets with ADP has been reported.(145)

The ability of nonsteroidal antiinflammatory drugs to inhibit collagen-induced aggregation was recognized a number of years before the involvement of arachidonate was suspected and the enzyme that these drugs affected was shown to be cyclo-oxygenase. In contrast, most of the drugs that inhibit thromboxane synthetase and prevent TXA_2 formation from PGH_2 were developed after the arachidonate pathway and the action of thromboxane synthetase were understood.(146) The majority of these drugs are derivatives or analogs of imidazole. Although they prevent thromboxane A_2 formation, they do not affect the formation of PGG_2 or PGH_2, which have also been identified as aggregating agents.(92, 93, 128, 147) Few studies of the effects of thromboxane synthetase inhibitors on platelet adhesion have been reported. Menys and Davies (148) have obtained data indicating that dazoxiben does not affect platelet accumulation on collagen-coated glass, but does inhibit platelet accumulation on damaged rabbit aorta, tested in vitro with ^{51}Cr-labeled rabbit platelets and a rotating probe.

Several investigators have used radioimmunoassays of thromboxane B_2, the relatively stable product formed from thromboxane A_2, as an indicator of platelet activation in vivo and in vitro. Although this is justifiable in vitro, the in vivo situation may be complicated by the fact that cells other than platelets (e.g., macrophages) produce thromboxanes (149) and thromboxane B_2 is cleared from the circulation so its concentration may not reflect episodic platelet activation. It should be emphasized that all the release-inducing agents that stimulate platelets activate the arachidonate pathway so that the demonstration of a high concentration of thromboxane B_2 in plasma gives no information about the agent that stimulated the cells to form it. It is certainly inappropriate to conclude that TXB_2 has been formed only by platelets adherent to an injury site, although if platelets have adhered they will contribute to the amount of TXB_2 in plasma.

Role of Platelet Ca^{2+}

There appears to be a close relationship between activation of the arachidonate pathway and activation of the release of platelet granule contents. Aggregating agents such as ADP, which do not cause the release of granule contents in a medium with a physiologic concentration of Ca^{2+}, do not cause the formation of thromboxane A_2. All of the release-inducing agents also activate the arachidonate pathway. It may be that a common reaction such as mobilization of internal platelet Ca^{2+} activates both processes. Many investigations have indicated that shape change, aggregation, and release of granule contents depend on an increase in the concentration of Ca^{2+} in the platelet cytosol from between 0.01–0.1 μM to between 1–10 μM, in most cases by mobilization of Ca^{2+} from sites of sequestration within the platelet.(150–160) These sites have not been determined, although suggestions include the dense tubular system (159, 161, 162) and the open canalicular system.(163) With the advent of the reagent "quin-2," it has become possible to determine the concentration of Ca^{2+} in platelets and the changes that occur when platelets are stimulated.(160) Although most aggregating agents cause an increase in the concentration of Ca^{2+} in the cytosol, Sanchez and Rink (164) have reported that collagen stimulates platelets without raising cytoplasmic Ca^{2+}. This observation is difficult to interpret, however, because platelets exposed to collagen release ADP, which does cause an increase in the concentration of cytosolic Ca^{2+}, according to Sanchez and co-workers.(165)

Stimulation of platelets with collagen appears to activate Ca^{2+}-dependent protein kinases in platelets, resulting in selective phosphorylation of specific proteins of mol wt 40,000 (P40) and 20,000 (P20).(156, 166) Phosphorylation correlates quantitatively with the extent of release of the contents of the amine storage granules, both of which occur even if aggregation is blocked by chelation of external Ca^{2+} with EDTA. One of the phosphorylated proteins, P20, is though to be the light chain of platelet myosin; phosphorylation of myosin light chain is necessary for actin-induced activation of myosin ATPase activity.(167) A calmodulin-dependent protein kinase is thought to be responsible for this reaction.(168) Phosphorylation and dephosphorylation of this myosin subunit are considered to regulate the contractile functions of platelets that are mediated by actin-myosin interactions and are activated when platelets adhere to collagen or are stimulated by other aggregating agents. Since PGE_1, which raises the concentration of cyclic AMP in platelets and thus causes the sequestration of cytosolic Ca^{2+},(169) decreased the collagen-induced phosphorylation of these proteins, it seems likely that the effect of collagen on the phosphorylation of these proteins is mediated by an increase in cytosolic Ca^{2+}.

It has been suggested that protein kinase C may be responsible for the phosphorylation of the 40K protein.(170, 171) This protein kinase is activated by diacylglycerol produced from phosphatidyl inositol, phosphatidylinositol 4-phosphate, or phosphatidylinositol 4,5-bisphosphate, and activation requires Ca^{2+} and phospholipid. However, it is active at physiologically low concentrations of Ca^{2+}. Collagen causes the formation of diacylglycerol and the phosphorylation of this 40K protein. Michell (171) has suggested that there is an intracellular synergism between the effects of elevated cytosolic Ca^{2+} and of activated protein kinase C in platelets.

Activation of the PI Cycle

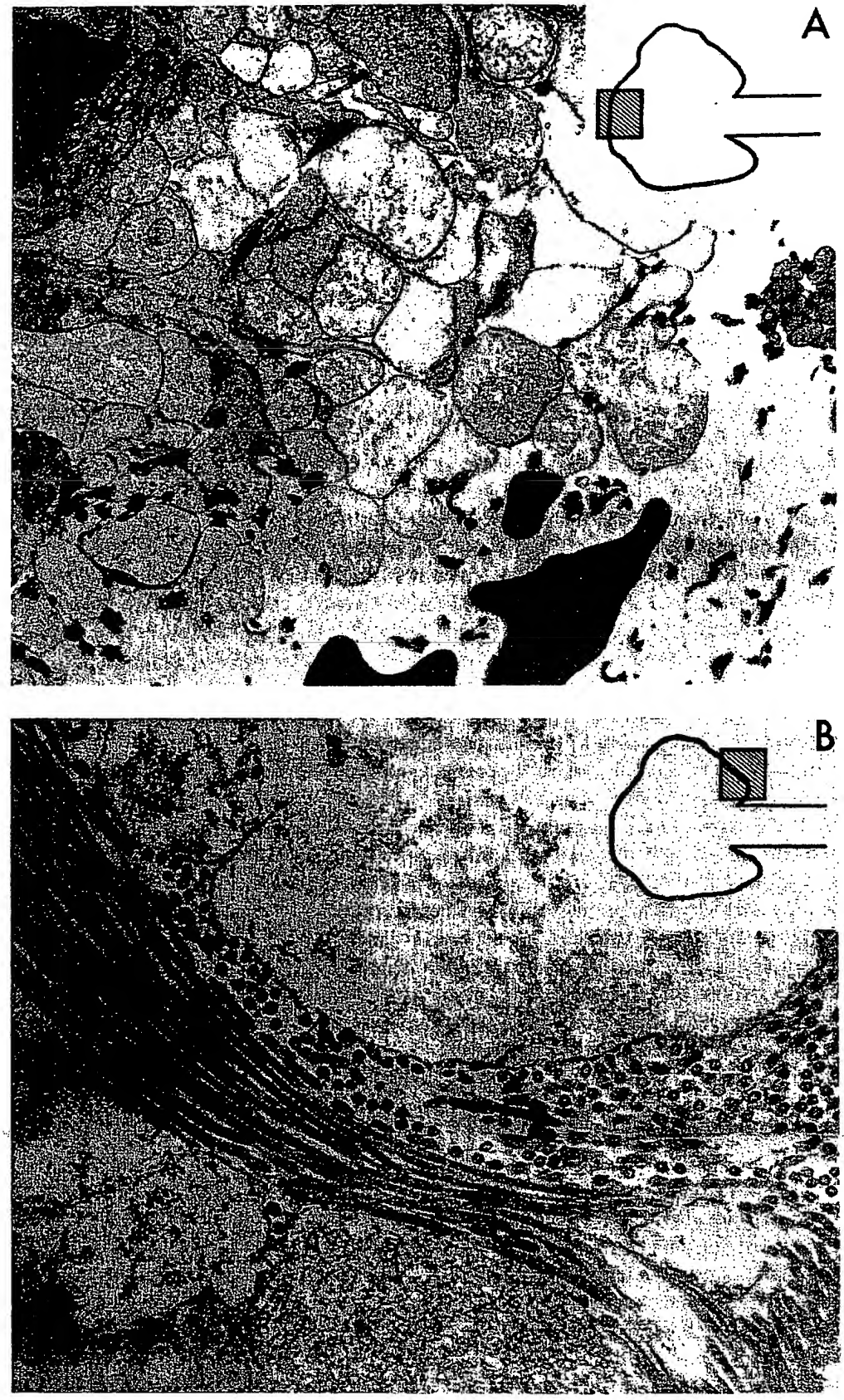
Another effect of collagen on platelets is activation of the phosphatidylinositol (PI) cycle in a manner that appears to be similar to its activation by thrombin.(172) This cycle is initiated by the action of phospholipase C, which causes the formation of inositol phosphates and 1,2-diacylglycerol from phosphatidylinositols.(130, 173–175) Diglyceride and monoglyceride lipases can free arachidonic acid from 1,2-diacylglycerol and it has been suggested that these reactions are important in freeing this precursor of thromboxane A_2.(131, 176, 177) If platelets are suspended in media that simulate physiologic conditions, however, little arachidonic acid is freed by this enzymatic pathway.(176, 178) Another proposal concerning this pathway is that 1,2-diacylglycerol may stimulate the Ca^{2+}-activated, phospholipid-dependent protein kinase that catalyses protein phosphorylation and results in the contractile reactions of stimulated platelets.(179) This is Ca^{2+} independent and phospholipase C is thought by some to not require Ca^{2+}.

How platelet adhesion to collagen stimulates a phospholipase, be it phospholipase C or phospholipase A_2, is not known. One of the consequences, however, is the freeing of arachidonic acid. To date, nearly all studies have been concentrated on the conversion of arachidonic acid to prostaglandins and thromboxane A_2 under the influence of cyclo-oxygenase and subsequently, thromboxane synthetase. Only recently has interest quickened in the effects of products formed from arachidonate if it is acted upon by lipoxygenase instead of cyclo-oxygenase in platelets. One of the products of the lipoxygenase pathway, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), is currently receiving attention because of the suggestion that it may promote platelet adhesion to collagen.(180) This theory is based on experiments comparing the effect of aspirin, which inhibits cyclo-oxygenase, with that of salicylate, which inhibits the formation of 12-HETE from 12-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE). Further testing of this hypothesis by other approaches is necessary before it can be accepted.

MECHANISM OF PLATELET ADHESION TO COLLAGEN

How platelets adhere to collagen is not established, although many studies have been done and several different theories have been advanced. Neither a receptor (if such exists) on the platelet membrane nor the components of the collagen fibers that take part in adhesion have been definitely identified.

Morphologic examination by electron microscopy of platelets adherent to collagen in hemostatic plugs and thrombi has shown that the distance between the surface membrane of the platelets and the collagen fibers is less than that between the surface membranes of aggregated platelets.(181, 182) A regular pattern of breaks in the membrane of adherent


A
B

platelets has been observed, matching the cross-striations of the collagen fibers.(70, 182, 183) In hemostatic plugs, platelets that are adherent to collagen are swollen and degranulated. In contrast, when adhesion to the subendothelium is complete, both transmission and scanning electron micrographs show that the platelets are flattened and spread on the surface, in addition to being degranulated (Fig. 1). (2, 5, 32)

STRUCTURE OF COLLAGEN AND PLATELET AGGREGATION

Collagen is composed of three helical polypeptide chains of equal molecular weight, called α-chains, linked by hydrogen bonds and intramolecular covalent crosslinks into a triple-stranded, coiled helix that is referred to as monomeric collagen or tropocollagen.(184–186) It has a mol wt of about 300,000.(187) Upon being warmed to 25°C, tropocollagen gradually polymerizes into soluble, microfibrillar collagen by intermolecular crosslinking. Further heating to 37°C results in the formation of larger fibrils (macrofibrils) that are insoluble.(187) The fibrils have also been described as "multimers," and the process of their formation as *multimerization* .(188) The term *quaternary structure* is also used.(187, 189)

At least five distinct types of collagen have been recognized, differing in amino acid composition and sequence, and in carbohydrate content.(186, 189, 190) Four of these types are found in vascular tissue (Table 1). The α1 chains in the various types of collagen differ from each other and are designated by the roman numerals assigned to the types of collagen. Type I collagen has two identical α1(I) chains in a triple helical conformation with one α2 chain $(\alpha 1(I))_2\alpha 2(I)$. Type II collagen is composed of three identical α1(II) chains, $(\alpha 1(II))_3$. Type III collagen has three identical α1(III) chains $(\alpha 1(III))_3$. Type IV collagen may also have three identical chains $(\alpha 1(IV))_3$ (189); although other investigators list its chain composition as "unknown,"(191) the most recent evidence indicates that it contains two genetically distinct α(IV) chains, (α1(IV) and α2(IV)).(186) Type V collagen contains α-chains that have been designated as A and B chains.(192, 193) The α-chains of types I, II, III, and V usually contain glycine in every third position, whereas type IV collagen has other sequences as well.(191)

Most studies of platelet aggregation have been done with type I collagen from tendon or skin. Soluble collagen has been prepared in neutral salt solution (188) or as acid soluble collagen (194) for studies of platelet adhesion and aggregation. Soluble monomeric collagen (tropocollagen) does not cause platelet aggregation,(188, 195) and tropocollagen does not bind to platelets to a measurable extent.(196, 197) The observation that monomeric collagen does not inhibit aggregation induced by chemically identical fibrillar collagen indicates that monomeric collagen may have a lower affinity for the platelet surface.(190)

Fig. 1. Electron micrographs of a hemostatic plug in a severed mesenteric vessel of a normal dog. The site from which the section was taken for examination is illustrated in the diagram of the plug in the upper right-hand corner of each picture. (A) At the periphery of the plug there are swollen platelet pseudopodia in contact with each other and with degranulated platelets. Interspersed among these platelets are dark patches of fibrin, many of them closely adherent to the surfaces of platelets. The large solid dark cells are red blood cells (magnification × 10,500). (B) At the point where the plug is in contact with the connective tissue around the vessel, the platelets are degranulated and swollen. The platelets are adherent to collagen fibers (magnification × 31,400) (from Mustard JF, Packham MA: Normal and abnormal haemostasis. Br Med Bull 33:187, 1977 with permission).

Table 1
Types of Collagen in Vascular Tissue

Type of Collagen	Types of Chains	Location*	Ability to Induce Platelet Adhesion
Type I	$(\alpha 1(I)_2\alpha 2(I)$	adventitia, media, subendothelium	yes
Type III	$(\alpha 1(III))_3$	media, subendothelium, adventitia	yes
Type IV	$(\alpha 1(IV))_3$ or $\alpha 1$ (IV) and $\alpha 2$(IV)	basement membrane	controversial
Type V	$(\alpha 1(V)_2\alpha 2(V)$	media, subendothelium, adventitia	controversial

Data from Weiss and Ayad (186); Barnes et al. (207); Rhodes (202)
* In decreasing order of abundance.

In contrast to the lack of effect of tropocollagen, multimers do bind strongly with a dissociation constant of less than 10 n*M*.(197) Muggli and Baumgartner (188) found a correlation between the development of a collagen structure capable of causing platelet aggregation, and the process of fibril precipitation. Simons and coworkers (198) observed that preformed collagen multimers were required for platelet aggregation. Kronick and Jimenez (197) also concluded that platelet-stimulating activity is not found in collagen solutions that are undergoing fibrillogenesis until well developed banded fibrils appear, but this can occur before the solution becomes visibly turbid. Some authors use the term "quaternary structure" to describe these fibrils that are reactive with platelets.(189, 199) However, Muggli (200) reported that the native quaternary structure is not necessary for platelet aggregation; he found that platelet aggregation could be induced by collagen fibrils with different quaternary structures. There is no doubt that much of the confusion and many of the apparent contradictions in the literature have arisen from the use of soluble collagen preparation in which fibril formation had occurred only partially, or was retarded by prior modifications of tropocollagen or by the conditions under which the collagen was incubated during fibril formation.(188, 195) Thus many of the reported observations are artefacts of in vitro experiments in which soluble collagen preparations were made for the purpose of purification, but steps were not taken to ensure that the collagen had regained its native state before its reactivity toward platelets was determined.

Vessel walls contain collagen of types I, III, IV, and V (Table 1).(192, 201) Type I is present mainly in the adventitia,(184) although smaller amounts are found in the media and subendothelium.(202) Types III and IV are present in the subendothelium; types IV and V are the collagen of the basement membrane. The initial belief that type III collagen is more reactive with platelets than type I collagen (203, 204) has been questioned because some of the differences have been shown to have been caused by variations in the rates of formation of fibrillar collagen in vitro.(190, 197, 205, 206) As noted earlier, monomeric collagen has little or no effect on platelets; the collagen triple helix must be assembled into fibrils before platelets will adhere.(188, 195) Gordon (205) has pointed out that although type III collagen appears to be more reactive with platelets than type I collagen when they are added to platelets in the monomeric form,(204) the difference practically disappears if preformed collagen fibrils are added.(207) The diameter of the fibrils also affects the amount of collagen required to cause platelet aggregation because it is surface area, rather than weight, that determines the extent of interaction with platelets.

Most investigators agree that type IV collagen, which is a component of basement membrane, does not cause platelet aggregation or the release of the contents of platelet granules.(192, 193, 208) Huang and Benditt (35, 209) have observed that platelets do not

adhere to the basal lamina after partial cleavage of its noncollagenous component by treatment with pepsin. They conclude that platelets do not adhere to collagen in the basal lamina, but rather, to the noncollagenous components of it, although this adhesion is not accompanied by loss of platelet granule contents. It seems likely that the primary structure of the chains of type IV collagen in basement membrane prevents the formation of fibrils with the highly ordered quaternary structure that characterizes the other types of collagen.(192) By electron microscopy, basement membrane collagen can be shown to lack any periodicity similar to the 67 nm banding that is characteristic of other collagens. Although type IV collagen has been reported by some investigators to interact weakly with platelets,(36, 201) other authors have questioned these findings and suggested that the type IV collagen preparations used may have been contaminated with type III collagen (35, 189, 209) see section on Basement Membrane.

Chemical Modifications of Collagen

A number of early experiments were done in which various prosthetic groups on collagen were chemically modified in attempts to determine those that are involved in platelet adhesion. For example, Wilner and coworkers (210) showed that acetylation of free carboxyl groups by treatment with methanol had little effect on the interaction between platelets and collagen, whereas blocking the epsilon-amino groups of lysine in collagen diminished its ability to cause platelet aggregation (and presumably its ability to adhere to platelets). Acetylation of the N and O groups of collagen by treatment with glacial acetic acid and acetic anhydride resulted in loss of the ability of collagen to cause platelet aggregation; this has been confirmed by Chesney and colleagues (211) for both soluble and fibrillar collagen. The situation became less clear, however, when Wilner and colleagues (212) went on to show that replacing the epsilon-amino groups with negatively-charged succinyl groups did not affect the ability of collagen to aggregate platelets, whereas esterification of the succinylated collagen strongly inhibited its ability to cause platelet aggregation. These observations led to the development of a theory that collagen must have rigidly spaced polar sites in order to interact with platelets, and that these sites may be either positively or negatively charged. The results with succinylated collagen have been questioned by Chesney and coworkers,(211) who found that succinylation of collagen completely abolished its ability to cause platelet aggregation, although the fibrillar structure was maintained. They pointed out that Nossel and colleagues did report about 75 percent inhibition of platelet aggregation in one of their studies in which they used succinylated collagen,(213) although later they reported no effect of succinylation.(212) They did find, however, that blocking the ϵ-amino groups of lysine with 2,4,6-trinitrobenzene sulfonic acid inhibited platelet adhesion to collagen as well as collagen-induced platelet aggregation.(212) Chesney and coworkers (211) also obtained contradictory results to those of Wilner and associates (210) regarding the effect of esterification of the carboxyl groups of collagen; Chesney and colleagues observed enhanced aggregation whereas Wilner and associates reported that acetylation of the free carboxyl groups did not significantly affect the platelet aggregating activity of collagen. In agreement with the early work of Wilner and associates,(210) however, Chesney and coworkers (211) concluded that ϵ-amino groups of lysine are of major importance in the interaction of platelets with collagen. This is in accord with the formation of bridges between platelets and collagen at the dense bands of collagen, which are thought to represent the polar regions of collagen molecules.(23)

In the experiments of Chesney and colleagues,(211) care was taken to determine whether the collagen was in the fibrillar of soluble form, and both types were modified and tested. Although some earlier studies may be criticized on the basis that modification of the side chains of the amino acid residues of collagen may have affected the formation of fibrils, rather than the interaction with platelets,(188, 205, 214) these objections are not relevant to the work of Chesney and associates.(211)

As pointed out by Santoro and Cunningham,(190) the modifications of collagen that have the greatest inhibitory effect on platelet adhesion are the modifications that reduce the positive charge on collagen. Although this might be expected because of the net negative charge on the surface of platelets, adhesion probably does not occur solely on the basis of charge, since red blood cells, which also have a net negative charge, do not adhere to collagen.

Role of Carbohydrate Side Chains of Collagen

The collagen glycosyltransferase theory of platelet adhesion that originally attracted a great deal of interest, is now known to be unacceptable. This theory was originally proposed by Jamieson and colleagues (215); it held that a glycosyltransferase on the platelet surface formed an enzyme-substrate complex with incomplete collagen-saccharide chains, since as many of 60 percent may be incomplete galactosyl-hydroxylysine.(187) This would normally be the first step in the transfer of glucose from uridine disphosphate glucose to galactose, although the completion of the reaction was not necessary for the theory.(216) Evidence against this theory includes the demonstration that collagens that lack galactosylhydroxylysine, or in which the carbohydrate has been altered, cause platelet adherence and aggregation.(184, 206, 217) Jamieson and coworkers (215) used the findings that glucosamine inhibited platelet adhesion to collagen, and inhibited collagen–glucosyl transferase activity in an assay using soluble collagen, as support for their theory. Legrand and colleagues, however, have shown that glucosamine inhibits the in vitro polymerization of soluble collagen, but has no direct effect on the interaction of platelets with collagen.(218) In keeping with their observations are the findings of Brass and Bensusan (199) that glucosamine does not affect platelet aggregation by collagen that is already in the fibrillar form. Glucosamine does not inhibit the adherence of platelets to a surface coated with fibrillar collagen.(219) The finding that treatment of collagen with galactose oxidase inhibits platelet adhesion to collagen (220) was originally used to support the theory, but the observation of Muggli and Baumgartner (188) and Harper and coworkers (221) that galactose oxidase delays polymerization of collagen would account for the effect of this enzyme. Further evidence against the collagen glucosyl–transferase theory came from the results of Menashi and coworkers,(222) who showed that synthesis of glucosylgalactosyl-hydroxylysine took place in the presence of the platelet enzyme when denatured collagen was used as substrate, whereas no synthesis occurred with native collagen. They concluded that adhesion of platelets to native collagen in vessel walls was unlikely to be mediated by collagen glucosyltransferase. Furthermore, they pointed out that the fact that the enzyme can be found not only on the membrane of platelets, but also in the cytosol and in high amounts in freshly prepared platelet-free plasma "casts further doubts" on its role in platelet-collagen adhesion. One would think that the collagen–glucosyltransferase theory would have been laid to rest several years ago, but is is still mentioned occasionally as if it were an acceptable theory.

According to several investigators, the sites on collagen required for interaction with

platelets do not appear to involve the carbohydrate portion of collagen.(206, 211, 217) Santoro and Cunningham (206) showed that the ability of types I, II, and III collagen to induce platelet aggregation does not correlate with their carbohydrate content, which differs widely. These investigators also showed that collagen that had been modified by periodate oxidation of its carbohydrate side chains, as well as the oxidized material that was subsequently reduced with sodium borohydride, were fully effective in inducing platelet aggregation.

It is of interest, however, that glycosylated collagen obtained from rats made diabetic with streptozotocin, or by nonenzymatic glycosylation of type I collagen from rats, is a more potent platelet aggregating agent than collagen from normal rats.(223) The amounts of bound glucose were 3 to 4 times greater in the glycosylated collagens and the conditions used lead to identical fibrillar structures of the collagens. Reactivity of platelets from diabetics to glass bead columns is also enhanced.(224) These observations lead to the suggestion that glycosylated collagen in the vessel wall may promote platelet interactions at sites of vessel injury to a greater extent than normal collagen does, and that this may play a part in the thrombotic complications of diabetes.

Telopeptide Regions of Collagen

There appear to have been no detailed studies of a possible role of the telopeptide regions of collagen in platelet adhesion, although they have been studied in relation to platelet aggregation and release. The telopeptide regions at both ends of the collagen molecule lack the typical polyproline structure that is involved in the formation of the triple helix. The telopeptides can be removed by treatment with pepsin, trypsin, or elastase, but this does not block the ability of collagen to cause platelet aggregation, providing fibrils form.(206, 210, 220, 225–227) Again, some of the contradictory results in the literature (227) may have arisen from experiments in which telopeptides were removed from soluble or monomeric collagen, but fibril formation from this modified collagen did not occur normally. Removal of telopeptides from monomeric collagen does interfere with its ability to form fibrils in plasma.(227)

Treatment of Collagen with Collagenase

It is to be expected that treatment of collagen with collagenase would destroy its ability to interact with platelets, and a number of investigators have demonstrated this.(28, 84, 210, 226) Baumgartner's group have used collagenase in a number of their studies of the interaction of platelets with the noncollagenous components of the subendothelium (32, 33) and others have also adopted this approach.(36, 228) When Chesney and associates (28) demonstrated that platelets contain a collagenase that can destroy the platelet-aggregating activity of collagen, they suggested that this enzyme may function as a negative feedback mechanism limiting thrombus formation. It is tempting to speculate that it may also be involved in the eventual loss of platelets from the surfaces of damaged vessels to which they have adhered and that the surface is altered by the degradation of collagen so that the surface becomes less thrombogenic.

Peptide Fragments of Collagen and Collagen-like Peptides

Several groups of investigators are studying fragments of collagen and other polypeptides to determine the parts of the collagen molecule involved in its adhesion to platelets.

Poly-L-hydroxproline has been shown to bind to platelets and to inhibit platelet adhesion to collagen.(145, 229) It has been suggested that the proline and hydroxyproline recognition sites on the platelet surface may not be used in adhesion to other surfaces.

The findings of Chiang, Beachey, Kang, and their coworkers are difficult to interpret.(230–233) They have reported that monomeric, purified α1I) chains of denatured chick skin collagen, and also a fragment from them [α1-CB5, a glycopeptide containing 36 amino acids and one residue of Glc-Gal-Lys(OH)], bind to platelets and cause platelet aggregation and the release of platelet granule contents. These findings are difficult to reconcile with the well established requirement for fibril formation for platelet-aggregating activity. Michaeli and Orloff (201) have suggested that some of the α-chains may have renatured before their effect on platelets was tested. Also puzzling are the observations of Beachey's group that α-chains of collagen from the skin of other species (rat, cow) or from other organs did not have the same effect as α-chains from chick skin, despite the close similarity in amino acid composition.(189)

In an extension of this work, Chiang and Kang (234) have reported the solubilization, purification by affinity chromatography, and characterization of a receptor on the platelet membrane for this α1-chain. It is a protein of apparent mol wt of 65,000.

Legrand and coworkers (235) have reviewed their own findings concerning cyanogen bromide fragments of type I collagen from calf skin.(236, 237) They have localized the site of adhesion to platelets on the C-terminal α1(I)CB6 peptide (216 amino acids) of type I collagen.

Fauvel and colleagues, working with Legrand, have published more detailed studies of the adhesion site on type III collagen.(235, 238–240) They first isolated a central α1(III) CB4 peptide of 149 amino acids by cyanogen bromide cleavage. This peptide was further degraded by treatment with chymotrypsin, hydroxylamine, and trypsin, and a nonapeptide (Gly-Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys) was identified as the segment responsible for adhesion of the α1(III) chain to platelets.(239) In further studies, these investigators have shown that a synthetic nonapeptide with this sequence inhibits platelet aggregation induced by type III collagen in vitro.(235, 241) Inhibition by this nonapeptide of aggregation of type I collagen was much less pronounced. Other aggregating agents were not affected by the presence of the nonapeptide. From these observations they have suggested that the adhesion of platelets to collagen may involve repetitive staggering of short amino acid sequences (such as this nonapeptide) along the rigid structure formed by a collagen fiber.(239)

More recently, Karniguian and coworkers (242) have identified an octapeptide from type III collagen (Lys-Pro-Gly-Glu-Pro-Gly-Pro-Lys) that is adjacent to the nonapeptide described above. When this peptide was synthesized, it was found to inhibit specifically platelet aggregation and release of granule contents induced by type III collagen, although it did not inhibit platelet adhesion to collagen. In these experiments, the observation that the octapeptide inhibited the increase in platelet cyclic AMP caused by PGI_2 is puzzling because this inhibitory effect is usually observed when platelets are exposed to aggregating agents, not to inhibitory agents.(243) The possibility exists, however, that the octapeptide may inhibit the interaction of PGI_2 with its receptor, although this was not suggested.

Multiple Interactions Between Platelets and Collagen

Despite the large number of investigations of the interaction of platelets with collagen, the precise nature of the binding is not known. Most investigators agree, however,

that collagen must be in the form of fibrils in order to bind to platelets,(196, 197, 244) to induce platelets to change shape,(245) to cause platelet aggregation, and to induce the release of granule contents.(188, 195, 198, 199, 211) As mentioned previously, the results of many of the early studies were misinterpreted because the treatments used to modify soluble collagen interfered with subsequent fibril formation, rather than with the adhesion of platelets to fibrillar collagen. It now seems to be established that neither the carbohydrate side chains of collagen nor the telopeptide regions are involved in platelet adhesion, whereas the ϵ-amino groups of lysine seem to be required.

Santoro and Cunningham (190) have advanced the theory that "multiple, simultaneous, linked interactions" are necessary for collagen-induced aggregation. They have pointed out that despite variations in amino acid sequence, fibrils of types I, II, and III collagen are all effective aggregating agents, although it is quite unlikely that these collagens are all capable of forming an identical, specific, multimolecular binding site. Like Muggli,(200) they have found that randomly crosslinked polymeric forms of collagen, which cannot form active type fibrils, can cause platelet aggregation. In their opinion, these observations eliminate the possibility that a macromolecular binding site recognized by the platelet is formed by the quarter-staggered arrangement of collagen molecules characteristic of the native type of collagen fibril. If their concept is valid, the inhibitory peptides described above must block the multiple sites required for the interaction of collagen with platelets.

Some of the wide variety of techniques used to measure the interaction of platelets with collagen will be described below. The conditions differ so markedly with respect to the medium in which the platelets were suspended (particularly the chelating agents present, or the concentration of Ca^{2+} and Mg^{2+}), the flow conditions, and the hematocrit, that it is often difficult to compare the results obtained by different investigators. Much more attention has been paid to the interaction of platelets with collagen because it was easier to study, than to their reaction with other constituents of damaged or diseased vessel walls, which may be equally, or even more important.

RECEPTOR ON PLATELETS FOR COLLAGEN

The nature of the receptor on platelets for collagen (assuming such a receptor exists) has not been established. Indeed, as noted above, Santoro and Cunningham (190) have proposed that, instead of an interaction with a specific, high-affinity receptor, multiple simultaneous and linked interactions occur between sites on the platelet surface and several sites on the collagen fibril. The signal for platelet activation would be the crosslinking of sites on the platelets by the collagen fibril. The receptor isolated by Chiang and Kang (234) for the 1-chain of chick skin collagen may not be a common receptor for other types of collagen. Nevertheless, the technique that they used of affinity chromatography to a peptide that reacts with platelets will undoubtedly be applied to the reactive peptides identified by Legrand's group in type III collagen, and more information about proteins or glycoproteins in the platelet membrane that interact with collagen will be obtained.

Lahav (246) used Sepharose® (Pharmacia) columns containing immobilized collagen to isolate adherent platelets, which were then lysed with Triton X-100. None of the proteins retained on the collagen was identifiable as a major membrane glycoprotein. In contrast, using the cross-linking reagent 3-3′ dithiobis (sulfosuccinimidyl propionate), and platelets labeled by the periodate, tritiated borohydride method, Kotite and coworkers (247) isolated two polypep-

tides that were crosslinked to collagen. In SDS gels, these polypeptides ran in the position of glycoproteins Ib and IIa. However, further characterization of the platelet polypeptides that become associated with collagen is required before the nature of the proteins or glycoproteins that are receptors for collagen can be determined.

It is well established that platelets from patients with the Bernard Soulier syndrome lack glycoprotein Ib on their membrane,(248–251) and that adherence to the subendothelium is defective.(252, 253) However, these platelets aggregate normally in response to collagen (254, 255) and it therefore seems unlikely that this glycoprotein is the receptor for collagen. Suggestions concerning reasons for impaired adhesion to the subendothelium and the possible role of von Willebrand Factor are discussed in the section, *Binding of von Willebrand Factor to Platelets*.

PLATELET ADHESION TO OTHER SUBENDOTHELIAL CONSTITUENTS

Although the interaction of platelets with collagen has been studied extensively, their reactions with the other individual components of the subendothelium have received relatively little attention. In addition to some fibrillar collagen with typical cross-striations, the subendothelium contains elastin, microfibrils around elastin, basement membrane and its associated proteoglycans, and a number of proteins (including laminin, fibronectin, von Willebrand Factor, and thrombospondin). The basement membrane is the main material present on the surface of the subendothelium of blood vessels. Much of the information about platelet adhesion to the structural components of the subendothelium comes from morphologic studies and has been reviewed previously by Stemerman.(26) The majority of the investigations have been done with rabbit platelets and rabbit aortas. In the few studies in which human vessels and platelets have been used, similar results were obtained despite the fact that the composition of the subendothelium of adult human arteries differs from that of young rabbits in that it is largely made up of smooth muscle cells with connective tissue between them.(256, 257)

Elastin and Microfibrils

Platelets appear to have little ability to adhere to elastin in the subendothelium.(2, 33, 258) This conclusion was derived in part from experiments with collagenase-digested subendothelium on which the initial deposition of contact platelets was similar to that on the subendothelium, but spreading was inhibited and only 5 percent of the surface was covered by platelets, whereas coverage of the subendothelium was 100 percent.(33) The platelets that did adhere lost their storage granules very slowly, in comparison with platelets adherent to a collagenous surface produced by digesting the subendothelium with α-chymotryspin. Baumgartner and colleagues (33) have classified elastin and its surrounding microfibrils as "virtually inactive" in inducing platelet degranulation.

Spaet and Erichson (71) found no adhesion of platelets to isolated elastin. Similarly, using isolated elastin and microfibrils in an EDTA-containing system, Barnes and MacIntyre (192) reported little adhesion of platelets to elastin (7 percent) compared to adhesion to type I collagen (78 percent), and even less adhesion to the microfibrils (1 percent). Elastin, therefore, appears to be relatively inert.

More recently, however, Fauvel and coworkers (34) have isolated a microfibrillar

extract of bovine aorta. This noncollagenous material caused platelet aggregation that depended on the presence of von Willebrand Factor, and was inhibited by a monoclonal antibody to glycoprotein Ib on the platelet surface. This direct evidence indicates that these microfibrils may play a part in the interaction of platelets with the subendothelium. Possibly the platelet interactions that did occur in the experiments by Baumgartner's group involved these microfibrils. In the early experiments in which platelets did not appear to adhere to isolated microfibrils, the requirement for von Willebrand Factor was not appreciated. It is not clear, however, whether von Willebrand Factor is required for adhesion, or for the subsequent release of granule contents and aggregation.

Basement Membrane

The basement membrane forms a thin sheet beneath the endothelial cells.(191) Type IV collagen, which has already been discussed, is a major structural component,(259–262) but it is not present in the form of fibrils and does not activate platelets. The main proteoglycan associated with the basement membrane is heparan sulfate.(262–264) Several glycoproteins are also localized on this structure.(262, 265)

Most studies of platelet reactions with basement membrane (basal lamina) have been done with glomerular basement membrane.(35–37, 209, 266) Although there is some disagreement, most investigators have found that platelets do not interact to an appreciable extent with type IV collagen, a major constituent of the basement membrane (see section on *Structure of Collagen and Platelet Aggregation*). Huang and Benditt (35) treated human glomerular basal lamina (HGBL) with either collagenase or pepsin. Removal of noncollagenous proteins with pepsin left a surface composed of residual collagen with little reactivity toward platelets. In contrast, after removal of collagen, platelet adhesion and spreading occurred to the same extent as on intact basal lamina, but no degranulation or aggregation was observed. Divalent cations were required for platelet adhesion to HGBL.(35, 266) In contrast, Freytag and colleagues (36) reported that both collagenous and noncollagenous components of bovine glomerular basement membrane caused aggregation of human or sheep platelets, but their observations have been criticized on the grounds that the systems were heterologous and that their preparations may have been contaminated with collagen fibers.(266) However, in a completely bovine system, Davis and associates (37) also observed adhesion of platelets to basement membrane; this occurred in the absence of added calcium, but release and aggregation did not take place until Ca^{2+} was added. It should be noted that the platelets were suspended in a most unsuitable medium containing 15 m*M* Tris buffer,(267) which may have affected their reactivity.

In one in vitro study of the reactivity of platelets in citrated blood or plasma with the basement membrane of rabbit heart valves, little or no platelet adhesion was observed.(268) In contrast, platelets have been observed to adhere to capillary basement membrane in vivo when endothelial cells have separated.(67–69, 269)

On the basis of the available information, it is not possible to decide whether the differences among experimental results are attributable to variations in experimental approaches, or are partly caused by differences in the reactivity toward platelets of basement membrane from different sources. Contamination with type III collagen has not always been ruled out in the preparations of basement membrane, and may account for some of the observations of aggregation and release of granule contents. Some of the discrepancies between in vitro and in vivo results may arise because of loss of loosely

associated components (proteoglycans, glycoproteins) during the isolation and purification of basement membrane preparations.

TECHNIQUES USED TO MEASURE PLATELET ADHESION

A great many methods have been described for quantifying platelet adhesion to collagen in vitro; this is a sure indication that none of them is entirely satisfactory. Nevertheless, some discussion of the different techniques seems warranted. They vary in many ways, including the platelet suspending medium, the method used to immobilize collagen, the type of collagen tested, and the method of quantitation. Platelet adhesion to the subendothelium has also been quantified by some of the same techniques. Table 2 outlines some of the reported methods.

The two main methods that can be used to quantify platelet adhesion either to collagen, or to the damaged surface of a blood vessel, are the morphometric and the isotopic methods. The most popular morphometric method was developed by and has been used extensively by Baumgartner and colleagues.(3, 32, 258, 270, 271) In this method, sections are prepared from blood vessels for direct examination by light microscopy or by scanning or transmission electron microsopy. Other investigators have adopted this method, or combined it with the isotopic method by perfusing the Baumgartner chamber with ^{51}Cr-labeled platelets.(136, 272) The advantages of the morphometric method are that both the adhesion of single platelets and of platelet thrombi can be determined separately and the area of the surface that is not covered by platelets can be estimated. In addition, adherent platelets that have contacted the wall but maintained their

Table 2
Methods used to Quantify Platelet Adhesion to Collagen or the Subendothelium

Method	References
Direct observation by light microscopy or by scanning or transmission electron microscopy of the surface to which platelets and thrombi have adhered	1-3, 5, 32, 33, 271, 276
Measurement of the amount of radioactivity associated with the surface after interaction with ^{51}CR- or ^{111}In-labeled platelets	5, 38, 136, 272, 273, 276
Enumeration of platelets before and after adhesion to surfaces coated with collagen or to columns of collagen/Sepharose	182, 225, 279, 280, 283
Measurement of change in light transmission as platelets adhere to a concentrated suspension of collagen	281, 282
Separation of collagen with adherent, labeled platelets from non-adherent platelets by:	
passage through Sepharose 2B	139
filtration on a membrane with 5 μm pores	285
Separation of ^{125}I-collagen adherent to platelets from unbound ^{125}I-collagen by centrifugation through Ficoll	196

The first two methods can be used in vitro or in vivo. For in vivo experiments, the experimental animals are exsanguinated and the vessels fixed by perfusion with a glutaraldehyde solution; fibrin formation during these procedures should be prevented by the prior administration of heparin. (5, 38)

shape, can be differentiated from platelets that have spread on the surface. The major disadvantages are the time-consuming and tedious nature of the process of preparing and examining the sections and the limited area that can be examined.

The main advantage of isotopic techniques is that the accumulation of platelets on a large surface area can be examined and measured easily and rapidly. The major disadvantage is that single, adherent platelets cannot be distinguished from platelet aggregates or thrombi on the surface. If aggregate formation on the adherent platelets is prevented or reversed, it is possible to measure accurately the number of platelets that are actually adherent to the surface. In the method originally described by Cazenave and colleagues,(194) the collagen-coated surfaces were merely rinsed in Tyrode solution, which would not have removed aggregated platelets. In later experiments, the surfaces were rinsed in EDTA to remove platelet aggregates and to leave only the platelets that were actually adherent to collagen.(273) This technique is suitable for rabbit platelets, which are readily deaggregated by EDTA, but it is unsuitable for human platelets, which are not deaggregated by EDTA if they have undergone the release reaction.(274)

A variety of devices has been used to study platelet adherence to collagen, the damaged surface of a blood vessel, or to artificial surfaces. The "Baumgartner chamber" has been mentioned earlier. In it, a central core is either coated with collagen or used as a mount for an everted vessel segment. In this annular chamber, blood or suspensions of platelets can be passed over the surface under controlled conditions of flow, and the shear rates can be adjusted to approximate those in vessels of various diameters.(275) One limitation is that blood vessels of different thicknesses cause variations in the distance between the core and the walls of the chamber and thus influence the shear rate.

Sakariassen and coworkers have developed a flat perfusion chamber to investigate the interaction of ^{111}indium-labeled platelets in flowing blood with human vessel wall cells in culture, their extracellular matrix, or isolated connective tissue components (collagen) at defined shear rates (276); they measured adhesion both morphometrically and by measuring radioactivity.

In the device used by Cazenave and colleagues, a cylindrical glass probe is coated with collagen or used as a mount for an everted vessel segment. The probe is rotated at constant speed for a predetermined time (usually 10 minutes) in a suspension of washed platelets to which a variety of additions can be made.(273) In the studies of Feuerstein and colleagues (277) and Cazenave and associates,(273) probes of this nature were rotated at low speeds (200 rpm) to give low shear rates (40 sec^{-1}, which is the shear rate in veins). In addition, materials released or formed by the platelets accumulate in the suspending medium during the time that the probe is rotated in the platelet suspension.

These two systems are suitable for measuring platelet adhesion to either a collagen-coated surface or the surface of a damaged vessel. With the exception of the system developed by Barnhart and associates (228) in which umbilical veins are used, most of the other devices developed to study platelet adhesion are not suitable for studying platelet adhesion to the subendothelium, but only to surfaces coated with collagen (or other proteins). These methods have been reviewed recently by Turitto and Baumgartner (275) and include flow chambers of flat plate, parallel plate, and stagnation point varieties. Since most of these have been used mainly to study platelet adhesion to artificial surfaces, they will not be discussed in detail in this chapter. It should be pointed out, however, that some investigators such as Muggli and associates (278) have used devices of this sort to investigate platelet interactions with a collagen-coated surface.

Several investigators have counted the number of platelets in suspension before and after adhesion has been allowed to occur.(182, 225, 279, 280) Spaet and Lejnieks (281) adapted the aggregometer to measure the small changes in light transmission that occurred as platelets in plasma that were anticoagulated with EDTA adhered to connective tissue fragments that were rich in collagen. Castellan and Steiner (282) used a variation of this technique. In all these studies, ETDA was used to prevent platelet aggregation. Unfortunately, EDTA or high concentrations of citrate partially inhibits platelet adhesion to collagen and strongly inhibits platelet adhesion to the subendothelium.(1, 97, 142, 273) The techniques employing EDTA permit the measurement of only the aspect of platelet interaction with collagen that does not require divalent cations in the suspending medium.

Brass and colleagues (283) have measured platelet adhesion to polymeric collagen that has been covalently linked to agarose (Sepharose 2B). Suspensions of washed radiolabeled platelets in a medium including Tris and EDTA were passed through a short chromatography column of collagen/Sepharose. Measurement of adhesion was based on ^{51}Cr labeling; measurement of release of granule contents was based on ^{14}C-serotonin released from prelabeled platelets. The major criticism of this technique is that EDTA must be used to prevent platelet aggregation in the column. In addition, Tris has been shown recently to inhibit collagen-induced platelet aggregation,(267) although its effect on platelet adhesion to collagen has not been investigated. Using the collagen/Sepharose system, Cowan and coworkers (142) have recently presented evidence that Mg^{2+} may have an important role in the adherence of platelets to collagen covalently linked to Sepharose. Shadle and Barondes (284) coupled collagen covalently to plastic slides and observed that adhesion was absolutely dependent on Mg^{2+}, whereas Ca^{2+} was ineffective. It should be recognized that this is an unusual system, however, since no platelet aggregation was observed in the presence of calcium, and PGE_1 did not inhibit adhesion. Thus measurements of adhesion to this surface may have little relation to adhesion to the subendothelium.

Legrand and coworkers (139) have devised a quantitative method for estimating platelet adhesion to fibrillar collagen, using platelets labeled with ^{51}Cr and ^{14}C-serotonin. A mixture of platelets and collagen is layered above a Sepharose 2B column, which retains the collagen fibrils with their adherent platelets and allows platelets and released serotonin to pass through. This technique suffers from the same problems as that of Brass and associates (283) in that the platelet suspending medium contains both EDTA and Tris.

In the method of Santoro and Cunningham,(285) ^{51}Cr-labeled platelets that were adherent to collagen fibers were separated on polycarbonate membrane filters with 5 μm etched pores. Again, EDTA was present in the suspending medium to prevent platelet aggregation.

Although a number of investigators have used platelets labeled with radioactive materials to study platelet adhesion to collagen and the subendothelium, the reverse of this technique has also been used. Gordon and Dingle (196) labeled the tyrosine residues of collagen with ^{125}I, added the labeled collagen to citrated platelet-rich plasma, and centrifuged the mixture through Ficoll. Platelets with bound collagen separated below the Ficoll, whereas collagen that was not attached to platelets did not penetrate. Their results indicated that collagen does not bind to platelets unless it is in the form of microfibrils. They they were able to establish that collagen microfibrils are required for the initial interaction of platelets with collagen, and not merely for the subsequent stage of collagen-induced aggregation.

Role of Ca^{2+} and Mg^{2+} in Platelet Adhesion

Many of the methods used to measure platelet adhesion have employed media containing EDTA to prevent platelet aggregation, despite the demonstrations that EDTA inhibits platelet adhesion. As pointed out by Turitto and Baumgartner,(275) it would be preferable if unanticoagulated whole blood could be used for studies of platelet adhesion, but the problems of platelet aggregation and the generation of thrombin in such a system have greatly limited this approach. In a few ex vivo studies, Baumgartner and colleagues (286–288) have studied adhesion using native blood (no anticoagulant) in their annular perfusion chambers, modified so that the blood was not recirculated. They found that platelet adhesion to the subendothelium was less in native blood than in blood anticoagulated with citrate, probably because fewer thrombi formed with citrated blood and thus more surface area was available for the adhesion of single platelets. Adhesion-induced aggregation was increased in native blood. In addition, higher shear rates were required in native blood to demonstrate decreased adhesion caused by von Willebrand Factor deficiency.(287) It may be that citrate, at the concentrations that are usually used to anticoagulate blood, does not have a major inhibitory effect on platelet adhesion to the subendothelium. It must be emphasized, however, that citrate also does not prevent aggregate formation on the adherent platelets.

Since some adherence of platelets to collagen and the subendothelium does occur in the presence of strong chelating agents, but platelet aggregation is blocked by EDTA or EGTA, many investigators have chosen to use these strong chelators so they could be sure they were studying only platelet adhesion without the confounding effect of aggregate formation on the adherent platelets.(139, 142, 281, 283, 289) Problems in the use of strong chelating agents has been pointed out in several sections of this chapter, since lack of divalent cations must be considered in the interpretation of results of adhesion experiments.

There appear to be differences in the requirements for adhesion to collagen and to the other constituents of the vessel wall; lack of divalent cations is more inhibitory of platelet adhesion to the subendothelium than to collagen.(1, 273) Little attention has been paid to the cation required (Ca^{2+} or Mg^{2+}). Using washed rabbit platelets suspended in Eagle's medium containing 2 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, 4 percent albumin, and blood cells to give a hematocrit of 40 percent, Cazenave and associates (273) found that 13 m*M* citrate, 4.5 m*M* EDTA, or 4.5 m*M* EGTA reduced platelet adhesion to a collagen-coated surface to 21 percent of control values, whereas these concentrations of chelating agents reduced adhesion to the subendothelium to less than 10 percent of control values. Using rabbit blood, Baumgartner's group (33) has shown that increasing the plasma concentrations of citrate from the usual 14.7 m*M* to 91.5 m*M* inhibits platelet adhesion to fibrillar collagen in the subendothelium and the formation of platelet aggregates, as does the use of EDTA at a plasma concentration of 5.7 m*M* (3 m*M* in blood). Surface coverage in 10 minutes was reduced from 80 percent (in 14.7 m*M* citrate) to 20 percent when chelation was increased in these ways. The ability of platelets to spread on the surface was also strongly impaired, but, nevertheless, both dense and α-granules disappeared, albeit at a slower rate, from the platelets that did adhere to fibrillar collagen, indicating that release from adherent platelets was not entirely dependent on divalent cations. In contrast to these observations, Kinlough-Rathbone and colleagues (97) observed that 5 m*M* EDTA blocked release of ^{14}C-serotonin from rabbit platelets that were adherent to the subendothelium, but so few platelets adhered in the presence of EDTA that a small extent of release would not have been detected.

Turitto and Baumgartner (275) have emphasized that, in the presence of EDTA, adherent platelets are predominately only in contact with the surfaces, rather than are spread out on them. Apparently only the contact phase is independent of divalent cations. Cazenave and associates have also observed that EDTA inhibits platelet spreading.(273) Much more extensive adhesion of platelets to collagen occurs in the presence of Ca^{2+}.(271, 275) It is likely that the inhibitory effect of EDTA is most apparent when there is a shear rate effect that would tend to remove platelets that have merely contacted the surface.

To study the effect of divalent cations on platelet adhesion, it is necessary to isolate platelets from plasma and to resuspend them in an artificial medium so that any possibility of thrombin formation is prevented when physiologic concentrations of divalent cations are present. If only adhesion is to be investigated, aggregate formation on the adherent platelets must be prevented, or the aggregates must be removed, leaving only the platelets that are actually adherent to the surface. Protein concentration, ionic composition, glucose concentration, pH and hematocrit should be similar to those in blood. In the system eventually devised by Cazenave and associates,(273) an enzyme system (apyrase) that degrades released ADP is also included to minimize the formation of platelet aggregates. As mentioned elsewhere, it is possible to remove aggregated rabbit platelets from the adherent platelets by rinsing the surface in a solution containing EDTA, but this is not effective with human platelets because they differ from rabbit platelets in that they are not deaggregated by EDTA if the release reaction has occurred.(274)

Effect of Red Blood Cells on Platelet Adhesion

There is general agreement that the presence of red blood cells increases the rate of platelet adhesion to collagen or to the subendothelium.(273, 291–295) Increased adhesion of platelets is attributed to two effects of red blood cells. First, in flowing blood they physically enhance the diffusion of platelets to the surfaces (277, 291, 293, 294, 296) and second, red blood cells may also contribute to platelet accumulation, on a surface if the red blood cells lose some of the ADP they contain.(293, 297–299) However, this ADP is likely to promote platelet aggregation, not platelet adherence. It has been shown recently that the large red blood cells of humans are more effective in promoting platelet adhesion to the subendothelium than are the smaller red blood cells of rabbits or goats,(295) and this is undoubtedly a physical effect.

Effect of Flow on Platelet Adhesion

The effect of blood flow on platelet adhesion has been the subject of numerous investigations; several comprehensive reviews have been published.(300–302) The rate of adhesion increases as the flow rate is increased.(3, 277, 303) In perfusion systems such as the annular Baumgartner chamber, this dependence holds for flow rates that give wall shear rates of less than approximately 650 sec^{-1}, the values typical of large veins and arteries.(275) Shear rates are highest in vessels of the smallest diameter. Turitto and Baumgartner (303) have pointed out that the adhesion rate is influenced by both platelet transport to the surface and by platelet reactivity with the surface. With shear rates above 1000 sec^{-1}, the latter effect may predominate. Most of these studies by Turitto and Baumgartner have been done under conditions of laminar flow.

In contrast, Goldsmith and Karino (301, 304) have done extensive studies of the

effects on platelet adhesion and aggregation of the disturbed blood flow that occurs around vessel orifices and downstream from stenotic-type obstructions in models of blood vessels. Their diagrams of reverse flow and vortices at such sites illustrate how platelets are trapped and accumulate, forming aggregates that diffuse to the wall and adhere to it. Their findings and theoretical analyses provide explanations for the early observations of the areas where platelets accumulated on the walls of arteriovenous extracorporeal shunts that contained bifurcations and branches.(305) The sites of platelet accumulation corresponded to the sites where atherosclerotic lesions are observed around vessel orifices, leading to the theory that platelet interaction with the arterial wall at sites of disturbed flow may an early event in the development of atherosclerosis.(305) This theory gained much more support when Ross and colleagues (306, 307) showed that platelet-derived growth factor (PDGF) is released from platelets when they interact with collagen, and that PDGF is mitogenic for smooth muscle cells.

As pointed out by Turitto and Baumgartner,(275) the role of blood flow in the removal of platelets from surfaces has received little attention. Many investigators have shown that platelet-thrombi can be dislodged,(1, 32, 303, 308, 309) but it is unlikely that the layer of platelets actually adherent to collagen or the subendothelium is ever removed in this way. Probably only the platelets that have aggregated on the adherent platelets are removed by the force of flowing blood.

ROLE OF PLASMA PROTEINS AND THROMBOSPONDIN IN PLATELET ADHESION

The roles in platelet adhesion of plasma proteins and proteins released from the α-granules of platelets are being investigated by many groups. The proteins of interest are those that have been shown to interact with the surface of stimulated platelets: von Willebrand Factor, fibrinogen, fibronectin, and thrombospondin. All of these are present in platelet α-granules, and all except fibrinogen are secreted by endothelial cells. In many studies, these proteins have been used individually to study their binding to platelets that have been isolated and resuspended in artificial media, often very unphysiologic media. Under these circumstances, the interactions among the proteins and their interference with, or promotion of the binding of, each other to platelets, or to injured vessel walls cannot be assessed. Since their concentrations in plasma vary widely, the situation in vivo may be quite different from that in the in vitro systems. At least two groups of investigators have demonstrated that fibronectin and thrombospondin interact with each other.(310, 311) Interactions with glycosaminoglycans in the subendothelium also deserve consideration.

Role of von Willebrand Factor in Platelet Adhesion to the Subendothelium

Although the statement has been made repeatedly in the literature that von Willebrand Factor is necessary for adhesion of platelets to the subendothelium of normal vessels, it is rarely pointed out that this is so only under certain defined conditions. First, von Willebrand Factor has a role in platelet adhesion only when the wall shear rates are high (greater than 1000 sec^{-1}).(275) When shear rates are similar to those in large arteries, that is in the range 500 to 1000 sec^{-1}, a deficiency of von Willebrand Factor does not affect the

number of platelets that adhere to the subendothelium either in vitro or in vivo.(312) In the coronary arteries of pigs deficient in von Willebrand Factor, removal of the endothelium did not result in decreased platelet adhesion to the subendothelium after a 30 minute period, in comparison to normal animals.(22) It is not surprising that the bleeding disorder in von Willebrand Factor deficiency is a disorder of smaller vessels where the shear rates are higher than in arteries.(275, 300) Second, in most assessments of the role of von Willebrand Factor in platelet adhesion to the subendothelium, citrated blood has been used. In this suspending medium, the effect of a lack of von Willebrand Factor can be shown at lower shear rates (500–1000 sec^{-1}) than in native blood in which no impairment of adhesion is present unless the shear rate is greater than 1300 sec^{-1}.(287)

In all the studies done by Baumgartner, Weiss, and their coworkers, subendothelial surfaces that may have considerably changed by storage in 0.2 *M* Tris buffer at 4°C for 2 to 4 weeks were used.(135, 287, 313) In early reports from this group,(135) penicillin and streptomycin were included in the storage medium, but it is not clear that they were used in later investigations. The effect that storage in this way may have on proteins that are normally present in the subendothelium (314) has apparently not been investigated; it is not known whether normal amounts of von Willebrand Factor remain after storage. Since adhesion of platelets at high shear rates is impaired in the absence of von Willebrand Factor in the blood used to perfuse the vessel segments, it must be concluded that in the experiments done by Baumgartner's group, insufficient von Willebrand Factor is present on the subendothelium of the segments to support platelet adhesion at a normal level. Information is not available on the question of whether fresh vessel segments would have enough von Willebrand Factor to promote a normal extent of platelet adhesion from blood deficient in von Willebrand Factor. Segments of human renal arteries obtained 12 hours after death that were prepared in Tris buffer have been found to bind ^{125}I-labeled von Willebrand Factor.(272) When platelets were suspended in a medium without von Willebrand Factor and perfused over these segments, the number of platelets that adhered was directly related to the amount of bound von Willebrand Factor. These observations indicate that the subendothelial surface prepared in this way lacks sufficient von Willebrand Factor to support the adhesion of platelets in normal numbers.

Nevertheless, some von Willebrand Factor must remain in the subendothelium prepared by the method of Baumgartner and associates, since pretreatment of the de-endothelialized surface of the rabbit aortas with antiserum to von Willebrand Factor diminishes the adhesion of human platelets in citrated blood to the surface.(315) From this observation Turitto and colleagues concluded that von Willebrand Factor in the vessel wall may be important in hemostasis. This is in accord with earlier findings of others (316, 317) who reported that von Willebrand Factor was lacking in the vessel wall of patients with severe von Willebrand's disease. The relative importance in platelet adhesion of von Willebrand Factor in the vessel wall and von Willebrand Factor in plasma has not been established.

Two groups of investigators have shown that von Willebrand Factor enhances platelet spreading on the subendothelium of human vessels in in vitro test systems.(318, 319) Although Baumgartner and colleagues,(320) using the subendothelium or the collagenous surface produced by digesting the subendothelium of rabbit aortas with α-chymotrypsin, concluded that decreased adhesion to fibrillar collagen was associated with normal spreading, their data indicate that spreading was defective with blood from patients with von Willebrand disease.(287, 320) Since one would expect spread platelets to be more strongly adherent than platelets that are merely in contact with the surface, enhancement of spreading by von Willebrand Factor would account for the observations that its effect

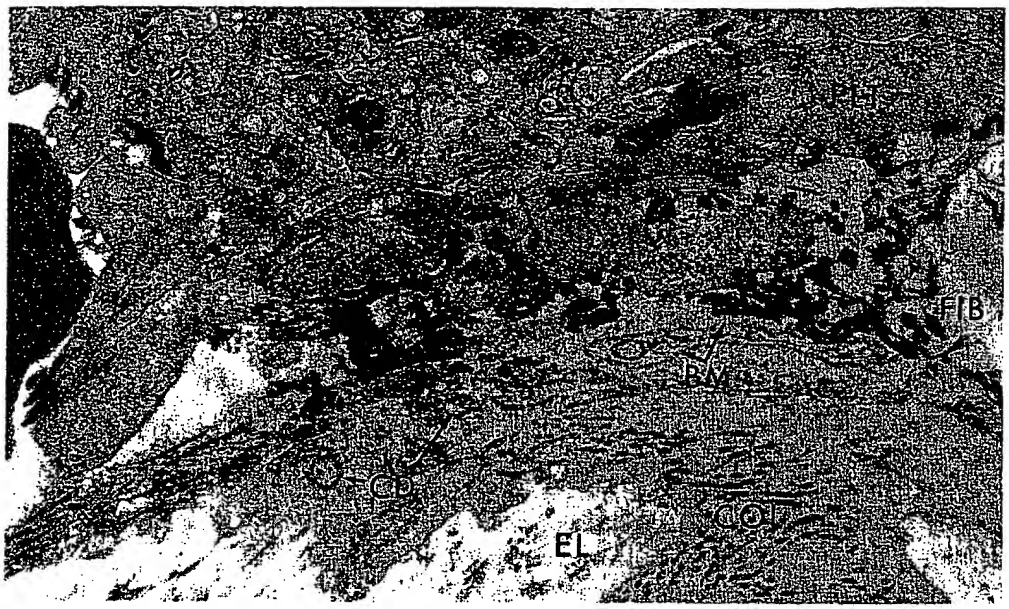


Fig. 2. The base of a thrombus in the aorta of a pig at the orifice of the second intercostal artery where blue staining was evident, following the administration of Evans blue. The thrombus rests upon basement membrane material (BM) and cellular debris (CD). Fibrin (FIB) predominates along the thrombus-vessel wall interface. EL, Elastic tissue; COL, collagen fibers; PLT, platelets (magnification × 17,200) (from Jørgensen L, Packham MA, Rowsell HC, et al: Deposition of formed elements of blood on the intima and signs of intimal injury in the aorta of rabbit, pig and man. Lab Invest 27: 341, 1972 with permission of *Laboratory Investigation*, ©1972 by the United States-Canadian Division of the International Academy of Pathology).

on platelet adhesion is only demonstrable at high shear rates, which would be less likely to remove spread platelets than "contact" platelets. More recently, however, the same group has reported a defect in the initial attachment of platelets from patients with von Willebrand's disease to de-endothelialized segments of vessel walls.(321) These results were obtained with blood that contained EDTA as an anticoagulant to inhibit platelet spreading, and subendothelium that had been digested with chymotrypsin. Since these investigators (1, 33) as well as others (273) have shown that EDTA strongly inhibits platelet adhesion to the subendothelium, it may be that lack of von Willebrand Factor affects only the aspect of platelet adhesion that is not dependent on divalent cations.

Somewhat different conclusions than those resulting from the use of the Baumgartner chamber have been reached by other investigators who have studied platelet adhesion in vivo in pigs that are congenitally lacking in von Willebrand Factor. In large arteries, such as the carotid arteries of these pigs, lack of von Willebrand Factor was found to have no significant effect on the number of platelets that adhered in vivo following injury by air drying, although platelet activation as indicated by spreading and degranulation was not observed.(322) Reddick and associates (22) also observed no difference in platelet adhesion to coronary arteries denuded of endothelium in normal pigs and pigs with von Willebrand's disease. These observations may be attributable to low shear rates in the vessels that were examined. As pointed out by Meyer and Baumgartner,(312) at low shear rates, other pathways for adhesion, not requiring von Willebrand Factor, may exist and be sufficient to support the adhesion of normal numbers of platelets. This concept is in keeping with the observations that platelets can cover the subendothelium or interact with

collagen at low shear rates without added von Willebrand Factor.(273) It also provides a rational explanation for the development of atherosclerosis and thrombosis in large vessels of patients with von Willebrand's disease,(323, 324) because if platelet adhesion were defective in these vessels, one would expect less stimulation of smooth muscle cell proliferation by the mitogen released from platelets at naturally-occurring sites of injury, and hence less atherosclerosis.

It seems reasonable to conclude that the major importance of von Willebrand Factor is in hemostasis involving small diameter vessels in which shear rates are high. Indeed, direct observation by electron microscopy of hemostatic plugs in patients with von Willebrand's disease reveals defective adherence of platelets to the severed vessel wall.(11, 325)

Methods of Quantitation of von Willebrand Factor Activity

The hemorrhagic disorder first described by von Willebrand (326) was later shown by von Willebrand and Juergens (327) to be associated with abnormal platelet function. They used a "capillary thrombometer" to demonstrate this abnormality. Measurement of the skin bleeding time is a nonspecific and imprecise, but generally reliable, way of detecting that an abnormality exists, and this method is still in use, although it may reflect not only abnormal platelet adhesion but also the low concentrations of Factor VIII that are characteristic of von Willebrand's disease. In the 1960s, columns packed with glass beads were developed to measure platelet adhesiveness upon passage of blood through them under defined conditions.(328, 329) Low values for platelet "adhesion" were observed with blood from patients with von Willebrand's disease. The addition of normal plasma or fractions of plasma containing von Willebrand Factor resulted in normal platelet "adhesion." Experimental conditions had to be rigidly controlled to obtain reproducible and valid results with this technique, and values tended to vary greatly among different laboratories. Other abnormalities influence platelet "adhesion" to glass beads, so the test is not specific for either von Willebrand Factor or indeed adhesion itself. The discovery that platelets from most normal individuals are agglutinated by the antibiotic ristocetin only if von Willebrand Factor is present (330) provided another test for von Willebrand Factor, although it is now known that platelets from about 30 percent of patients with von Willebrand's disease agglutinate normally with ristocetin.(331) Several types of von Willebrand's disease are known, and in type IIB agglutination of platelets by ristocetin is greater than normal.(332) An additional complication is that the ristocetin test is not specific since platelets from patients with the Bernard Soulier syndrome also do not agglutinate in response to ristocetin.(333, 334) There are some conditions in which ristocetin cofactor activity (VIII R:RCo) does not correlate with the bleeding time, which may remain prolonged after VIII R:RCo has been corrected by infusion of Factor VIII concentrates.(332) In addition, Bowie and colleagues (335) have obtained evidence, from studies with several different monoclonal antibodies directed against von Willebrand Factor, that the part of the molecule involved in ristocetin cofactor activity is not identical to the parts that determine the bleeding time.

The participation of von Willebrand Factor in platelet adhesion to the subendothelium at high shear rates has been demonstrated with the perfusion chamber developed by Baumgartner and colleagues in which blood is passed over everted segments of de-endothelialized rabbit aorta and the number of adherent platelets is determined morphometrically.(32, 287) Other investigators have used human renal arteries with ^{51}Cr-labeled platelets (256, 272, 318) or umbilical veins.(228) Platelet adhesion is defective with

blood from patients with von Willebrand's disease or reconstituted blood lacking von Willebrand Factor,(272, 287, 334, 336) and can be corrected by the addition of von Willebrand Factor (256, 272, 337)

The von Willebrand Factor protein can be assayed by several immunologic techniques involving specific heterologous antibodies, quantitative or radioquantitative immunoelectrophoretic techniques, or immunoradiometric assays. The literature concerning these has been reviewed in detail by Zimmerman and Ruggeri.(332) These authors point out that single quantitation of the antigen may not give a measure of the true von Willebrand Factor protein activity because von Willebrand Factor exists in plasma in the form of multimers of various sizes with different von Willebrand Factor activities. Although the small multimers are measured by the quantitative immunologic techniques, they do not have the von Willebrand Factor activity that is required for normal hemostasis. When the large multimers are lacking, as they are in types IIA and IIB von Willebrand's disease, the concentration of Factor VIII-related antigen may be normal, as measured by these assays, although the bleeding times are prolonged. These assays are information, however, for type I von Willebrand's disease, in which the amount of von Willebrand Factor protein is reduced, but the multimeric composition is normal, and for type III, in which von Willebrand Factor protein is practically undetectable in plasma by sensitive radioimmunoassays.(338)

Role of Large M_r Multimers of von Willebrand Factor in Platelet Adhesion

As indicated above, von Willebrand Factor consists of a series of multimers ranging in molecular weight from approximately M_r 800,000 to over M_r 12×10^6,(332) perhaps as large as M_r 20×10^6.(339) The ability of the multimers to correct the hemostatic defect in von Willebrand disease is directly related to their size. In type IIA and type IIB von Willebrand disease, the large multimers are absent from plasma, and the bleeding time is prolonged even if high amounts of the small multimers are present. It seems likely, therefore, that it is the large multimers that take part in platelet adhesion to connective tissue at the ends of severed blood vessels in normal individuals. Furthermore, the large multimers bind preferentially to platelets on which von Willebrand Factor receptors have been exposed by treatment of the platelets with thrombin,(340) the large multimers take part more readily in ristocetin-induced agglutination of platelets,(341–343) and they bind preferentially to fibrillar collagen.(344)

Unusually large multimers of von Willebrand Factor have been found in the plasma of some patients with chronic relapsing thrombotic thrombocytopenic purpura (TTP).(345) During relapses, in vivo platelet agglutination and thrombocytopenia were associated with decreased quantities of these large multimers in plasma, in comparison with their amounts during remission. Moake and colleagues (345) suggested that the large von Willebrand Factor multimers may be "consumed" during relapse (presumably by taking part in the formation of platelet aggregates that are removed from the circulation), and that the abnormality responsible for the presence of the abnormal multimers in these patients may be a defect in processing after their synthesis and secretion by endothelial cells. They related their findings to the platelet agglutination activity detected in the plasma of some patients with TTP (346, 347); normal plasma inhibits this agglutinating activity in vitro.(346) It seems likely that these large multimers may play a part in the thrombotic episodes in this condition.

In the recently described "pseudo" von Willebrand's disease, the patients have a mild

bleeding disorder, intermittent thrombocytopenia, decreased amounts of Factor VIII/von Willebrand Factor in their plasma, and a deficiency of the high molecular weight multimers in their plasma.(339, 348–351) As in type IIB von Willebrand's disease, ristocetin-induced agglutination of platelets is enhanced; this has been attributed to a platelet membrane abnormality that results in the adsorption to the platelets of high molecular weight multimers. The increased binding of these multimers to platelets may deplete plasma of these multimers and may be responsible for the thrombocytopenia.(339) The observation that the abnormal platelets are aggregated by normal human von Willebrand Factor is in accord with this interpretation. Lack of the large molecular weight multimers in plasma in this disorder is likely to be responsible for the bleeding diathesis.

Binding of von Willebrand Factor to Collagen

Several groups of investigators have demonstrated that von Willebrand Factor is adsorbed by collagen.(344, 352–357) Legrand and associates (353) reported that type III collagen, which is found in the subendothelium, bound von Willebrand Factor much more effectively than type I collagen. This difference between the types of collagen was subsequently confirmed by Nyman.(352) According to Santoro and Cowan,(355) binding of von Willebrand Factor to fibrillar type I collagen from skin is independent of temperature and does not require divalent cations or other plasma proteins. A preferential binding by collagen of the higher molecular weight forms of von Willebrand Factor has been observed.(344) In view of these findings and the observations to be discussed later that stimulated platelets can bind von Willebrand Factor, it is not surprising that adsorption of von Willebrand Factor on the surface of exposed collagen at a site of vessel injury is considered to be involved in platelet adhesion to damaged vessel walls.

Role of von Willebrand Factor in Platelet Adhesion to Collagen

Although the participation of von Willebrand Factor in platelet adherence to the subendothelium under conditions of high shear rates is well established,(272, 287, 336) the possibility that von Willebrand Factor has a role in platelet adhesion and spreading on a collagenous surface is not entirely settled. Most investigators have reported that platelet aggregation induced by collagen is normal in patients with a deficiency of von Willebrand Factor (254, 255); these observations indicate that although the direct interaction of some platelets with collagen must occur during collagen-induced aggregation, von Willebrand Factor may not be essential for this interaction. It should be pointed out, however, that Baumgartner and colleagues (313) found that antibodies to von Willebrand Factor that had been prepared in rabbits, as well as homologous antibodies, inhibited platelet adhesion to collagen fibrils at high shear rates. No inhibition was apparent at low shear rates, but with increasing shear rates, inhibition also increased until at shear rates that are typical of the microvasculature (1300–5200 sec^{-1}) platelet adhesion was practically abolished by the antibodies. The collagen fibrils were actually the exposed surface of α-chymotrypsin-digested subendothelium, and other materials are undoubtedly present on this surface. The rabbit antiserum directed against human Factor VIII:vWF also inhibited ^{14}C-serotonin release induced by platelet adhesion to collagen in EDTA-PRP in an aggregometer cuvette in which stirring was rapid.(358) As much as 70 percent inhibition was observed. The investigators suggest that the vWF antigen–antibody complexes could sterically hinder collagen fibrils from gaining access to their receptors on the platelets, thereby directly interfering with the adhesion of platelets to collagen.(358) To reconcile their observations with the fact that collagen-induced aggregation of platelets from patients with von Wille-

brand's disease is normal, they pointed out that inhibition by the antiserum was small and significant only on a paired basis and apparent only with low collagen concentrations.(358) Santoro and Cowan (355) also found that an antibody to human von Willebrand Factor, raised in goats, inhibited platelet adhesion to collagen in the absence of added von Willebrand Factor, although von Willebrand Factor released from the platelets may have been present on their surface. By using fluorescein-conjugated rabbit antiserum to goat immunoglobulin, these investigators showed that the anitbodies to von Willebrand Factor that inhibited adhesion were bound to the platelet surface. They suggested that the interactions of multiple von Willebrand Factor molecules on the platelet surface with multiple sites on the collagen fibril could give rise to high affinity adhesion despite the rather weak individual interactions.(206, 355) In the studies by Santoro and colleagues (206, 355), and that of Morin and coworkers,(359) who showed that platelet adhesion to collagen was diminished in von Willebrand's disease, platelet adhesion was studied in the presence of EDTA to prevent platelet aggregation. As discussed elsewhere in this chapter, platelet adhesion to collagen in a medium containing EDTA is much less than in a medium containing physiologic concentrations of Ca^{2+}, under conditions in which platelet aggregates are not present.(142, 273) It may be that von Willebrand Factor affects only the small part of platelet-to-collagen adhesion that is not dependent on divalent cations. The findings with antibodies to von Willebrand Factor are not readily reconciled with the observations that suspensions of washed platelets that have been induced to release their granule contents by treatment with thrombin, and presumably have lost their releasable von Willebrand Factor, are readily aggregated by collagen.(360) Most of the evidence indicates that at low shear rates von Willebrand Factor appears to be unnecessary for platelet adhesion to collagen, although it does have a role at high shear rates.

Role of von Willebrand Factor in Interaction of Platelets with Noncollagenous Microfibrils

Fauvel and coworkers (34) have produced a noncollagenous microfibrillar extract of bovine aorta that caused platelet aggregation; this interaction was diminished by a deficiency of von Willebrand Factor in the plasma, or by a monoclonal antibody to glycoprotein Ib on the platelet surface, indicating that there may be another thrombogenic constituent of the vessel wall that is not collagenous and is dependent on von Willebrand Factor for its interaction with platelets. It is not clear, however, whether von Willebrand Factor is required for platelet adherence to these microfibrils; it may be necessary only for the induction of the release of platelet granule contents, which leads to platelet aggregation.

Binding of von Willebrand Factor to Platelets

Glycoprotein Ib and Ristocetin

In the Bernard Soulier syndrome, glycoprotein Ib on the membrane of platelets is absent or reduced in amount (246–249) and platelet adhesion to the subendothelium is impaired, resulting in prolongation of the bleeding time.(252, 253) Platelet aggregation in response to collagen is normal.(255) As in von Willebrand Factor deficiency, the effects of the lack of glycoprotein Ib are most apparent in vitro under conditions of high shear rates, according to Weiss and associates.(287) Caen and colleagues,(361) however, demonstrated that a human IgG developed in a Bernard Soulier patient inhibited human

platelet adhesion to rabbit subendothelium at low shear rates.(362) Platelets from patients with the Bernard Soulier syndrome also fail to agglutinate in response to the antibiotic ristocetin in plasma containing normal amounts of von Willebrand Factor.(333,334) Several groups of investigators have shown that in the presence of ristocetin, von Willebrand Factor binds to a receptor on the surface of normal platelets, and that this binding is impaired with platelets from patients with the Bernard Soulier syndrome.(363–365) These findings have led to the theory that glycoprotein Ib may function as a receptor for von Willebrand Factor on the platelet surface. However, ristocetin is not present in vivo, and no molecule with a similar function has been identified in blood or tissues, so the physiologic significance of the binding of von Willebrand Factor induced by ristocetin is not clear. Nevertheless, Legrand and coworkers (366) have suggested that in the Bernard Soulier syndrome there is a defective interaction between platelets and the microfibrils in the subendothelium, in which von Willebrand Factor normally takes part by binding to glycoprotein Ib on the platelet membrane. A monoclonal antibody to human platelet glycoprotein I has been shown to inhibit platelet interactions with microbifrils and with collagenase-treated subendothelium.(367)

Binding of von Willebrand Factor to Stimulated Platelets

It has been suggested that stimulation of platelets by aggregating and release-inducing agents such as ADP, thrombin, or collagen may be involved in making a receptor for von Willebrand Factor available on the platelet surface. Specific binding sites for von Willebrand Factor have been reported to become available on the surface of platelets that have been treated with thrombin.(104, 340) George and Onofre (105) have shown that platelets suspended in an artificial medium release von Willebrand Factor in response to thrombin, and bind the released protein by means of a calcium-dependent mechanism. They suggest that this may provide a way in which platelets can concentrate and organize their secreted proteins for subsequent physiologic reactions such as hemostasis. Nachman (368) has suggested that subendothelial proteases may act like thrombin and induce von Willebrand Factor–receptor function in adhering platelets.

However, the observations of Ruggeri and colleagues (103) with platelets from patients with Glanzmann's thrombasthenia provide evidence against the theory that the receptor for von Willebrand Factor that is exposed by thrombin treatment of platelets is involved in platelet adhesion to the subendothelium. They found that the specific binding of von Willebrand Factor to thrombin-stimulated platelets from thrombastenic patients is less than 20 percent of normal;(103) this is an indication that von Willebrand Factor binds to thrombin-stimulated normal platelets through glycoproteins IIb and IIIa. Nevertheless, thrombasthenic platelets adhere in undiminished numbers to the subendothelium,(320, 369) although they do not aggregate in response to any aggregating agent, and they agglutinate and bind von Willebrand Factor in the normal way in response to ristocetin.(103, 370, 371) Thrombasthenic platelets lack glycoproteins IIb and IIIa, which results in their inability to bind fibrinogen and aggregate when they are activated by aggregating agents.(372) Since the adherence of thrombasthenic platelets to the subendothelium is not impaired if the amount of von Willebrand Factor in plasma is normal,(320, 369) it is questionable whether the binding of von Willebrand Factor to the receptor exposed by thrombin treatment of platelets (assuming this receptor is the glycoprotein IIb/IIIa complex) is involved in the adhesion of platelets to the subendothelium.

Ruggeri and coworkers (106) also point out the difficulty of explaining why binding of von Willebrand Factor to thrombasthenic platelets stimulated with ristocetin is normal,

whereas binding to these platelets after they have been exposed to thrombin is abnormally low. It seems likely that under certain conditions, binding of von Willebrand Factor to platelets can occur in two different ways. Glycoprotein Ib has been identified as the binding site on platelets for von Willebrand Factor in the presence of ristocetin (373) and platelets from patients with Glanzmann's thrombasthenia have normal amounts of glycoprotein Ib.(374) Another possibility is that glycoproteins IIb and IIIa are involved in binding von Willebrand Factor to thrombin-treated, normal platelets, and that this binding differs from that involving glycoprotein Ib and ristocetin.

At least two attempts have been made to determine the relative contributions of these two binding sites. Green and Muller (375) found that 70 to 90 percent of labeled von Willebrand Factor bound to platelets during ristocetin-induced agglutination, whereas only 21.5 percent bound to platelets aggregated with thrombin (2.5 U/ml). Using suspension of washed platelets in a medium that did not contain any proteins, Harrison and McKee (376) also quantitated the binding of labeled von Willebrand Factor to platelets stimulated with ristocetin or thrombin. The amount of von Willebrand Factor that bound to platelets treated with thrombin in their experiments was only 6 percent of the amount that bound to platelets exposed to ristocetin. However, in neither of these studies was the release of platelet α-granule contents measured. If the thrombin caused more release of von Willebrand Factor than did ristocetin (which seems likely), the released von Willebrand Factor would dilute the labeled von Willebrand Factor, resulting in deceptively lowered estimates of binding to thrombin-stimulated platelets.

Thrombin is not the only aggregating agent that induces the binding of von Willebrand Factor to platelets. Fujimoto and Hawiger (377, 378) have reported that treatment of platelets with ADP or the ionophore A23187 also leads to increased binding of von Willebrand Factor to them. Lahav and Hynes (379) have observed that when platelets adhere to collagen and spread on the surface, they become able to bind von Willebrand Factor, probably in association with the release reaction induced by the platelet-collagen interaction, which reveals binding sites on the surface of platelets. Di Minno and coworkers (380) have reported binding of "Factor VIII" (undoubtedly von Willebrand Factor) to platelets stimulated by arachidonic acid, collagen, U-46619 (a stable analog of prostaglandin endoperoxide/thromboxane A_2), thrombin, or ADP.

Recent observations that von Willebrand Factor does not bind to ADP-stimulated or thrombin-stimulated platelets in the presence of fibrinogen at concentrations normally present in plasma, although it binds in the absence of fibrinogen,(381) cast doubt on a role for von Willebrand Factor binding to the glycoprotein IIb/IIIa complex under physiologic conditions. It is evident that binding studies in the absence of other plasma proteins may yield deceptive results.

There appear to be at least two sites on platelets with which von Willebrand Factor can interact, namely glycoprotein Ib and the glycoprotein IIb/IIIa complex. A monoclonal antibody against glycoprotein Ib had no effect on the binding of von Willebrand Factor to platelets stimulated with thrombin or a combination of ADP with epinephrine, whereas a monoclonal antibody against the glycoprotein IIb/IIIa complex had no effect on ristocetin-induced binding of von Willebrand Factor to normal platelets.(106) It seems likely, however, that glycoprotein Ib is a binding site involved in platelet adhesion. Evidence against a major role for the glycoprotein IIb/IIIa complex is that thrombasthenic platelets, which lack this complex, adhere to the subendothelium in essentially normal numbers, and von Willebrand Factor does not bind appreciably to the complex in the presence of fibrinogen. Since the adhesion of platelet lacking glycoprotein Ib is impaired at high shear

rates, binding of von Willebrand Factor at this site is probably involved in platelet adhesion under normal conditions.

Role of Fibronectin

There has been considerable interest in the possibility that fibronectin may be involved in the binding of platelets to collagen, or to other constituents of the subendothelium. One group of investigators (382) has suggested that it may even be the receptor for collagen on platelets, but this seems unlikely. It is well established that fibronectin can bind to collagen.(382–385) Indeed, fibronectin is such an adhesive protein that it binds to almost everything with which it comes in contact.(386) Its role in cell adhesion and spreading in other systems has been demonstrated repeatedly. The domain of fibronectin that binds to collagen does not mediate cell interactions, however.(386) The questions are, does fibronectin bind to collagen under in vivo conditions when collagen is exposed, and if so, to what extent does this binding promote platelet adhesion? The alternative possibility, that fibronectin on the platelet surface takes part in adhesion to collagen seems less likely because very little fibronectin is present on unstimulated platelets (387) to take part in the initial stages of adhesion. A role in the later stages, however, cannot be ruled out.

As pointed out by Hynes,(388) three sources of fibronectin are available to platelets when the subendothelium is exposed. Fibronectin is synthesized by endothelial cells, and large amounts are found in the basement membrane (387); plasma contains fibronectin at a concentration of 300 μg/ml;(388) and the α-granules of platelets contain fibronectin.(110, 379, 389–392)

Fibronectin Binding to Stimulated Platelets

Unstimulated platelets, and presumably the normal, circulating platelets, do not have appreciable amounts of fibronectin bound to their surface.(110, 385) When platelets are stimulated with thrombin, however, released fibronectin becomes bound to their surface (110, 387) and fibronectin added to the suspending medium will also bind to the platelet surface.(393) This binding is inhibited by EDTA.(393) Stimulation of platelets with ADP or epinephrine apparently does not reveal these fibronectin-binding sites,(393) but interaction with collagen may do so.(379, 390) In the experiments of Zucker and colleagues (390) with collagen, it was not established that fibronectin-binding sites became available; these investigators showed only that collagen caused the release of fibronectin and that aspirin inhibited its release. The results with aspirin indicate that thromboxane A_2 was probably responsible for the release of fibronectin, but the effect of thromboxane A_2 (or its precursor, arachidonic acid) has not been tested to determine whether stimulation of platelets in this way will make fibronectin-binding sites available. Fibronectin binds in undiminished amounts to thrombin-stimulated platelets from patients with the gray platelet syndrome.(394) Since these platelets are deficient in α-granule contents, including fibrinogen and thrombospondin, it is unlikely that binding of fibronectin is mediated solely by fibrin or thrombospondin.

It seems probable that any agent that causes the release of platelet granule contents will reveal the binding sites for fibronectin since the release reaction in response to thrombin and the appearance of fibronectin on the surface occur at similar rates.(111) If thrombin were found to be the only stimulus that would make these sites available (and this seems unlikely), then in order for platelets to adhere to collagen through bound fibronectin, thrombin would have to be generated at a site of vessel injury before fibronec-

tin could bind to the platelet surface. A more reasonable hypothesis is that the surface of platelets that adhere to collagen is altered so that fibronectin can bind to them, and that this fibronectin takes part in the spreading of platelets on collagenous surfaces.(379, 395–397)

Site of Fibronectin Binding to Platelets

The site on the surface of stimulated platelets to which fibronectin binds has not been definitely identified although there have been some investigations directed at determining its nature. (The possibility that thrombospondin may have a role is discussed later.) A fibronectin-binding glycoprotein has been isolated from human platelet membranes.(398) The observations of Ginsberg and associates (399) that thrombasthenic platelets, upon stimulation with thrombin, bind very little fibronectin (either fibronectin released from the platelets or fibronectin added to the suspending medium) points to a role for the complex of glycoproteins IIb and IIIa that becomes available when platelets interact with aggregating agents. This complex has been recognized as the receptor for fibrinogen on the surface of stimulated platelets and it has also been suggested as a binding site for von Willebrand Factor as a result of similar experiments with thrombasthenic platelets.(103) Whether or not fibronectin and von Willebrand Factor bind to this glycoprotein complex in the presence of normal plasma concentrations of fibrinogen has not been established, nor indeed, has the effect of fibrinogen been studied on the binding of fibronectin to normal platelets that have undergone a release reaction. This type of experiment could not be done with thrombin, of course, and it would have to be shown that other strong release-inducing agents make the fibronectin-binding sites available before the effect of fibrinogen on the binding could be assessed.

The complex of glycoproteins IIb and IIIa of aggregated platelets has been shown to be attached to the platelet cytoskeleton (400) and fibronectin can bind to actin.(401) Thus the demonstration by Bensusan and associates (382) that when washed platelets were reacted with collagen fibers and then sonicated, fibronectin and the contractile proteins myosin, actin, and tropomyosin remained attached to the collagen, may indicate that the fibronectin took part in the binding via attachment to the complex of glycoproteins IIb and IIIa, although these glycoproteins were not identified in the mixture after sonication.

Effect of Fibronectin on Platelet Adhesion to Collagen

Bensusan and colleagues (382) have suggested that fibronectin is the collagen receptor on platelet membranes. There are now several pieces of evidence indicating that this is unlikely. Santoro and Cunningham (285) have demonstrated that treatment of platelets with purified antibody or Fab′ fragments to fibronectin only slightly reduces platelet adhesion to collagen, and Sochynsky and coworkers (402) also found that pretreatment of platelets with antibodies to fibronectin did not inhibit the adhesion of platelets to collagen. As Mosher (389) has pointed out, the sequence of the α1(III) chain of type III collagen that has been shown to be involved in platelet adhesion to collagen (239) does not resemble the sequence of the site on type I collagen that is recognized by plasma fibronectin. Very little fibronectin is present on unstimulated platelets (110, 387) to take part in the initial stages of adhesion; platelets suspended in artificial media without fibronectin, however, do adhere to purified preparations of collagen, and fibronectin released from the platelets could be involved in the later stages of spreading of platelets on the surface.(395–397) It will be of interest to find out whether gray platelets that lack α-granules, and therefore presumably lack releasable fibronectin, will adhere normally to collagen in a plasma-free system. This possibility does not seem to have been investigated. The experi-

ments of Reimers and associates (360) with platelets that were degranulated with thrombin do not provide a definitive answer, although these platelets had presumably released their fibronectin. These platelets aggregated normally in response to collagen and adhered to a collagen-coated surface, but the possibility cannot be ruled out that released fibronectin remained bound to their surface and took part in the interaction with collagen.

Several groups of investigators have reported that preincubation of collagen with fibronectin interferes with the interaction of collagen with platelets; these findings have been interpreted in different ways. Bensusan and colleagues (382) used them as evidence that fibronectin on the platelet surface was involved in the adhesion of platelets to collagen, and that this platelet fibronectin could not bind to collagen to which fibronectin was already attached. In contrast, Moon and Kaplan (403) took the observation that added fibronectin prolonged the lag time of collagen-induced aggregation to support a theory that plasma fibronectin was an inhibitor of the reaction of platelets with collagen. Sochysnsky and coworkers,(402) on the basis of results similar to Bensusan and associates,(382) concluded that plasma fibronectin would inhibit the adhesion of platelets to collagen. In contrast, Chazov and colleagues (404) have reported that fibronectin enhances the adhesion and spreading of platelets on a surface coated with fibrillar collagen.

Although the report that fibronectin corrected defective collagen-induced aggregation in a relative with a variant of Ehlers-Danlos syndrome has been taken as support for the theory that fibronectin is required for the interaction of platelets with collagen,(405, 406) the platelets from these patients were abnormal in other ways.(405) For example, they did not undergo the second phase of aggregation in response to ADP in citrated platelet-rich plasma. Since fibronectin is not required for ADP-induced aggregation,(390, 407) the failure of these platelets to aggregate in response to collagen may not be entirely attributable to a lack of functional fibronectin.

What is to be concluded from these studies of fibronectin? There is no doubt that fibronectin binds to collagen and binds to the surface of stimulated platelets. It is unlikely, however, to be the "receptor" for collagen on the platelet surface, or to take part in the initial interaction of platelets with collagen. Fibronectin may be involved in platelet spreading on collagen and firm attachment to it. If so, its role may be limited and there may be other adhesion mechanisms that are equally or more important.(285) The role of fibronectin in platelet adhesion to collagen in the presence of normal plasma concentrations of the other proteins that may compete for its binding site on platelets remains to be determined. A possible role for fibronectin in platelet adhesion to other constituents of the vessel wall has received little attention, probably because of the technical difficulties of such studies.

Role of Fibronectin in the Interaction of Platelets with Fibrin

The possibility of a role for fibronectin in platelet adhesion to fibrin has not been explored, although it is known that fibronectin becomes covalently crosslinked to fibrin during clotting (408) and that platelets adhere to polymerizing fibrin (15) and are required for clot retraction. During fibrin formation, the platelets are stimulated by thrombin, and their fibronectin-binding sites would become available, as well as their fibrinogen-binding sites, regardless of whether or not these sites are identical. It is not known whether crosslinking of fibronectin to fibrin enhances the adhesion and spreading of platelets as it does fibroblasts.(409) Several interactions could take place simultaneously. These interactions could be of importance in the adhesion of platelets to repeatedly damaged vessels, or

to diseased vessels, where thrombin and fibrin appear to have major roles in the accumulation of platelets.(17, 38)

The Role of Thrombospondin in Platelet Interactions with Collagen

Thrombospondin has been identified as the lectin-like material that becomes available on the surface of platelets that have released their granule contents under the influence of strong release-inducing stimuli such as thrombin or the divalent cation ionphore A23187.(107, 109, 410–412) Thrombospondin appears to be the α-granule protein that has been identified by Phillips and colleagues (108) as glycoprotein G, and earlier, by Baenziger and associates as thrombin-sensitive protein.(413) It has been suggested that thrombospondin on one platelet may form links with fibrinogen on an adjacent platelet to contribute to platelet aggregation in response to release-inducing agents.(107, 311) Thrombospondin is synthesized and secreted by endothelial cells (414, 415) and fibroblasts.(416) Since thrombospondin can bind to fibronectin (310, 311) and both of these proteins are present at sites of vessel injury, it may be that thrombospondin on the surface of activated platelets can bind to fibronectin that is attached to collagen or is present in the subendothelium. As mentioned previously, however, fibronectin binds in normal amounts to thrombin-stimulated platelets from patients with the gray platelet syndrome that lack thrombospondin and fibrinogen.(394) This observation indicates that thrombospondin on the surface of activated platelets is not essential for the binding of fibronectin to them.

Role of Complement in Platelet Interactions with Collagen

Although complement bound to the platelet membrane does not appear to be involved in platelet adhesion to collagen, it may have a role in the subsequent events of release of granule contents, and aggregation.(417) Platelets from dogs decomplemented with cobra venom factor do not aggregate in response to collagen, but do adhere to collagen fibers.(417) Platelets from C6-deficient rabbits aggregate normally in response to collagen,(418) but platelets from guinea pigs lacking C4 do not.(419) The platelets from the C-4 deficient animals, however, adhere normally to collagen.(419) Antisera to C1, C3, and C5 was also shown to inhibit collagen-induced platelet aggregation, but adhesion was not studied under these conditions.(417) One theory that has been advanced is that collagen to which platelets are adherent may activate their membrane-bound complement,(417) since collagen has been shown to activate C1.(420) (See section on "Inhibitors with Structural Similarities to Collagen" for a discussion of the effects of C1q and C1s.)

ROLE OF PROTEOGLYCANS IN THE INTERACTION OF PLATELETS WITH COLLAGEN

Little attention has been paid to the fact that in vivo, collagen is usually coated with proteoglycan,(421, 422) which undoubtedly modifies its interaction with platelets and affects its binding with fibronectin to which some proteoglycans also bind.(386, 389, 422–425) Heparin, highly sulfated heparan sulfate and hyaluronic acid have been reported to enhance the interaction of fibronectin with collagen.(426–428) Proteoglycans can also interact with thrombospondin.(429) Methods used to extract collagen from tissue (such as

extraction with high molar salt solutions) remove proteoglycans so that their effects are not present in the in vitro test systems of platelet adhesion or aggregation.(423) Rich and coworkers (430) have shown that proteoglycans from cartilage inhibit the adhesion and spreading of fibroblasts on collagenous surfaces, but similar experiments do not appear to have been done with platelets. The few reports concerning proteoglycans, collagen, and platelets are concerned with collagen-induced aggregation rather than adhesion. Muir and Mustard (431) showed that a chondroitin-4-sulfate-peptide potentiated collagen-induced platelet aggregation, but other investigators have reported inhibition by high concentrations of chondroitin sulfate.(23) Zucker-Franklin and Rosenberg (23) found that platelets did not adhere to unextracted bovine or human cartilage and that cartilage did not aggregate platelets, but after removal of proteoglycan by extraction with 3 *M* guanidine hydrochloride, the collagen that remained caused aggregation. They isolated a proteoglycan subunit that inhibited collagen-induced aggregation, but did not affect ADP-induced aggregation. Since this subunit also inhibited polylysine-induced agglutination, they suggested that platelet interaction with collagen may involve suitably spaced polar groups on collagen and platelets rather than a specific receptor on the platelet surface. More recently, Ts'ao and Eisenstein (423) showed that preincubation of human collagen from skin with proteoglycan from bovine cartilage inhibited the ability of the collagen to aggregate human platelets, whereas preincubation with proteoglycan from bovine aorta did not. It is apparent that the information currently available is insufficient to permit the development of a clear picture as to the possible role of proteoglycans in regulating platelet interactions with collagen or other components of the subendothelium.

ENZYMATIC MODIFICATIONS OF THE PLATELET SURFACE

A few investigators have examined the effect of modification of the surface of platelets on their ability to adhere to collagen and on collagen-induced aggregation of platelets. Removal of over 50 percent of the sialic acid residues on the platelet surface by neuraminidase treatment does not inhibit platelet adherence to a collagen-coated surface and does not diminish, but slightly enhances, the extent of collagen-induced aggregation.(432) Adherence to the subendothelium is also unaffected by treatment of platelets with neuramindase.(89, 432) Treatment of platelets with periodate to oxidize terminal sialic acid residues, however, diminishes platelet adherence to collagen and the subendothelium, and inhibits collagen-induced aggregation.(433) It must be emphasized, however, that periodate undoubtedly oxidizes other components of the platelet membrane and, in this way may affect the ability of platelets to adhere to collagen. In the experiments of Cazenave and associates, pretreatment of platelets with the proteolytic enzymes thrombin, plasmin, chymotrypsin, and trypsin reduced platelet adhesion to a collagen-coated surface or to the subendothelium.(137, 434) The pretreatment of the platelets with chymotrypsin (which did not cause release of amine storage granule contents) diminished the extent of platelet aggregation and release of granule contents caused by collagen, but did not prevent the responses completely, although the PAS-staining of glycoprotein I was completely abolished, indicating that the PAS-staining glycopeptide of glycoprotein I had been removed by the treatment with chymotrypsin.(435) Santoro and Cunningham,(285) using a different assay system, also found that treatment of platelets with chymotrypsin reduced their ability to adhere to collagen by more than 50 percent. More recently, however, Lahav and Meyer (436) reported that pretreatment of platelets with chymotryp-

sin did not affect platelet adhesion to collagen fibers coated onto a glass surface. One can only speculate that these contradictory results are attributable to the differences in the systems used to measure platelet adhesion or the extent of the pretreatment with chymotrypsin.(435) Cazenave and colleagues (137, 273) used platelets in a Ca^{2+}-containing medium with apyrase to degrade released ADP and a hematocrit of 40 percent; after adhesion had taken place, the surfaces were rinsed in EDTA to remove platelets that might have aggregated on the platelets that had adhered to collagen. Lahav and Meyer (436) used platelets suspended in a solution containing EGTA to prevent platelet aggregation. Chelation of Ca^{2+} with EGTA and EDTA is known to inhibit platelet adhesion to a large extent,(1, 33, 273, 434) so these investigators may have been studying only part of the platelet-adhesion reactions. Chelation of divalent cations cannot provide a complete explanation for the discrepancy, however, because Santoro and Cunningham (285) studied adhesion in a medium containing EDTA and obtained results with chymotrypsin-treated platelets that agree with those of Cazenave and associates.(137, 434)

A similar discrepancy was observed with thrombin-treated platelets. Cazenave and coworkers found partial inhibition of platelet adhesion to collagen or the subendothelium,(137, 434) whereas Lahav and Meyer (436) reported no inhibition of adhesion to collagen fibers. Both groups agreed, however, that pretreatment of platelets with trypsin decreased the ability of platelets to adhere to collagen.

EFFECT OF PLATELET AGE ON PLATELET ADHESION

A possible relationship between the changes of platelet membrane glycoproteins that may occur as platelets age, and their ability to adhere to collagen has not been established. Hirsh and colleagues,(290) using rabbit platelets labeled in vivo with ^{35}S to obtain labeled populations of young or old platelets, found that the young platelets preferentially adhered to collagen in platelet-rich plasma anticoagulated with EDTA. They also demonstrated that the infusion of collagen into rabbits with young ^{35}S-labeled platelets caused a fall in the specific radioactivity of the circulating platelets, whereas infusion into rabbits in which the ^{35}S-labeled platelets had been allowed to age did not change the specific radioactivity of the circulating platelets. They concluded that young platelets adhered to collagen more readily than older platelets. Although Spaet and Lejnieks (281) interpreted their own results, showing that all the platelets in suspension adhered to collagen, as an indication that platelets of all ages could adhere, the two studies are not necessarily contradictory. Spaet and Lejnieks (281) used a large excess of collagen whereas Hirsh and colleagues (290) used a lesser amount so that all of the platelets did not adhere. This permitted them to identify the ones that adhered less readily as the older platelets. Since these early studies, Castellan and Steiner (282) have reported that young platelets adhere more readily to collagen fibers than randomly aged platelets.

PLATELET ADHESION TO FIBRIN

The ability of platelets to adhere to fibrin has been recognized for some time, because of evidence arising from morphologic examination of hemostatic plugs and thrombi, and from the phenomenon of clot retraction. Hemostatic plugs and thrombi are stabilized by the fibrin that forms on their surface and among the aggregated platelets at the periphery

of the plug or thrombus.(10, 41, 70) This fibrin is formed under the influence of thrombin generated at the surface of platelets that have undergone the release reaction; platelet factor 3 becomes available on their surface and accelerates two steps of the intrinsic coagulation pathway, leading to local formation of thrombin. Platelets appear to adhere to fibrin as it polymerizes (15, 437, 438); they do not adhere to fully polymerized fibrin if the thrombin that caused its formation has been neutralized.(182) The interaction of platelets with polymerizing fibrin is inhibited by EDTA, but adhesion of platelets to the polymerizing fibrin is not inhibited by agents such as PGE_1 or apyrase, which inhibit aggregation. Electron micrographs have shown platelets adherent to fibrin in retracting clots (439, 440); in some sections, platelets appear to have engulfed fibrin particles.(440)

Several investigators have observed fibrin between platelet aggregates and damaged vessel walls in deep injuries, or on repeatedly injured sites where a neointima composed of smooth muscle cells has formed.(18, 38–41) The conclusions from these findings are that thromboplastin from the damaged cells accelerates the extrinsic coagulation pathway and that, as fibrin polymerizes, it adheres to the damaged cells; platelets in the blood flowing past the polymerizing fibrin then adhere to it, so the plug or thrombus is initiated by platelet adhesion to the polymerizing fibrin adherent to the injured vessel wall.

The fact that activated platelets and polymerizing fibrin are required for clot retraction is strong evidence that platelets adhere to polymerizing fibrin during this process. Clot retraction occurs under the influence of thrombin in whole blood, in platelet-rich plasma, or in suspensions of isolated platelets, provided fibrinogen is present. It seems likely that the fibrinogen receptor on platelets is involved in their interaction with fibrin because thrombasthenic platelets, which lack the glycoprotein IIb/IIIa complex that, with Ca^{2+}, constitutes this receptor, are unable to cause clot retraction.(441) In addition, if this reversible receptor is not made available on the platelets, clot retraction does not occur. This has been demonstrated in experiments in which fibrin has been formed under the influence of reptilase, which does not stimulate platelets; unless the platelets in the clot are stimulated by an aggregating agent such as ADP or arachidonic acid, which makes the fibrinogen receptor available, clot retraction does not occur.(437, 442–445) Inhibitors such as PGE_1 that prevent the fibrinogen receptor becoming available in response to aggregating agents,(112, 446) also inhibit clot retraction.(447, 448)

The in vivo equivalent of clot retraction is the consolidation of hemostatic plugs and thrombi. The adhesion of polymerizing fibrin to platelets and other cells such as fibroblasts, leukocytes, and endothelial cells (437, 449) is probably important in repair processes after vessel wall injury. It has also been suggested that clot retraction may pull the sides of a wound together or facilitate recanalization of thrombosed vessels by pulling the clot away from one side of the vessel wall, and based on in vitro experiments, a possible role in clot lysis has received recent support.(450)

It is evident that under circumstances in which fibrin formation plays a major part in the initiation of thrombi, drugs that inhibit the generation of thrombin or prevent its action on fibrinogen and platelets are likely to be more effective in limiting thrombus formation than drugs that affect only platelet function.

PLATELET ADHESION TO WHITE BLOOD CELLS

Normally, platelets do not interact with leukocytes in vivo. However, as pointed out by Needleman and Hoak,(451) the abundance of these two cell types in white bodies is

too great to result from passive adsorption. These authors suggest that their mutual affinity for polymerizing fibrin (437) may be responsible for their occurrence together, or that chemotactic factors formed or released by platelets may attract leukocytes.(452–455) Alternatively, leukocytes may further platelet adhesion by damaging the endothelium.(451)

Platelets have been observed adherent to the large number of macrophages that become associated with the vessel wall lesions of animals given hypercholesterolemic diets for prolonged periods of time.(55)

In the rare abnormal condition variously termed platelet satellitism, granulocyte–platelet rosette formation, platelet neutrophil adherence, or platelet-to-leukocyte adherence pheonomena (PLAP), platelets in plasma anticoagulated with EDTA adhere to polymorphonuclear leukocytes and are phagocytosed by them.(456–464) The rosettes are not observed in blood that does not contain an anticoagulant, so any in vivo significance is obscure. PLAP has been described in association with thrombocytopenia,(458) with an inherited platelet defect that is similar to that of storage pool-deficient platelets,(464) with diabetes,(463) with Behcet's disease,(457) with thrombocytopenic purpura associated with malignant lymphoma,(462) and with several other diverse conditions, but no consistent pattern related to specific disease states has emerged. The abnormality appears to be caused by a factor in plasma in some cases,(462) and in others, by a platelet abnormality.(463)

PLATELET INTERACTIONS WITH TUMOR CELLS

Platelets are aggregated by some tumor cells and by membrane fragments isolated from these cells.(465–469) It is apparent that platelet adhesion to the tumor cells must occur, but this aspect of the interaction does not appear to have been examined in detail. Aggregation is demonstrable in heparinized, but not in citrated platelet-rich plasma, and a requirement for Mg^{2+} has been identified.(468) According to Donati and associates,(466) the binding of tumor membrane fragments or vesicles to platelets requires activation of the first four components of the complement system. It has been suggested that platelet aggregation then results from the generation of thrombin by the tumor-vesicle-platelet complex.(470)

ADHESION TO PARTICULATE MATERIALS AND PHAGOCYTOSIS BY PLATELETS

Early observations of the ability of platelets to adhere to and, in some cases, phagocytose particulate matter have been reviewed previously.(471) Among the materials that have been investigated are thorotrast, polystyrene (latex) particles, ferritin, viruses, fat particles, silicone dioxide, colloidal carbon, antigen–antibody complexes, malarial parasites, and a number of types of bacteria.(406, 472–488)

Many investigators have studied the phagocytosis of latex particles by platelets. In citrated platelet-rich plasma, these particles adhere to platelets, are found in invaginations in the membrane and, within a few minutes, can be observed within the platelets.(471, 481) This process causes the release of the contents of platelet granules and results in platelet aggregation.(471, 489, 490) White (480) has emphasized, however, that uptake of

latex particles by platelets differs from phagocytosis by leukocytes, in that bacteria enter neutrophils in sealed vacuoles derived from the cell wall, and the products deposited in the vacuoles during neutrophil degranulation remain in the vacuoles and are not released to the exterior.

Inhibitors of aggregation (AMP, adenosine) do not interfere with phagocytosis by platelets (471), nor do inhibitors of cyclo-oxygenase that prevent the formation of thromboxane A_2,(481) but metabolic inhibitors do inhibit phagocytosis of latex particles.(475, 478, 479) In early studies, it was observed that EDTA greatly diminished phagocytosis and prevented aggregation, although it did not prevent the adherence of the particles to the platelets, nor the release of granule contents.(471, 475, 491) These observations were taken as indicating that divalent cations are required for phagocytosis, but not for adhesion. More recently, Lewis and colleagues (481) reported that they observed no difference in the extent of phagocytosis of latex particles in plasma from blood anticoagulated with citrate or EDTA. Zucker-Franklin (482) also was able to study phagocytosis in the presence of EDTA. The reason for this discrepancy concerning a requirement for divalent cations is not clear. With isolated platelets in artificial media, coating the latex particles with fibrinogen increased adhesion in comparison to uncoated, or albumin-coated particles, but had little effect on the release of platelet constituents, whereas coating the particles with γ-globulin resulted in extensive release of the contents of platelet granules.(489) This observation is undoubtedly related to similar events resulting from the phagocytosis of antigen–antibody complexes by platelets.(476) Lewis and coworkers (481) have concluded that the phagocytosis of latex particles by platelets is chronologically similar to that reported for polymorphonuclear leukocytes. This conclusion is based on their observations that there was a progressive accumulation of the particles in the open canalicular system of platelets, followed by localization in electron-opaque vacuoles; after 60 minutes, acid phosphatase (a lysosomal granule marker) was localized within the latex-containing vacuoles, indicating that these vacuoles are phagosomes, whereas these vacuoles did not stain with alkaline-bismuth, which stained the external membranes and the membranes of the open canalicular system. Lewis and associates (481) suggested that the sequence of events is adhesion of the particles to the surface of the platelets, sequestration into the open canalicular system, and finally the formation of phagocytic vacuoles through a process requiring metabolic energy.(475, 478, 479) These conclusions differ from the earlier suggestion of White (480) that most of the latex particles remained in channels of the open canalicular system that are not pinched off to form sealed phagocytic vacuoles.

On the basis of electron microscopy of freeze-fractured platelets, Zucker-Franklin (482) has proposed that large particles (such as latex particles) are taken up in a different fashion from small particles, although both must adhere to platelets initially. She has suggested that large particles are taken up by membrane invaginations that are apparently independent of the pits believed to represent entrances to the open canalicular system; this process is thus similar to phagocytosis by leukocytes, and would require metabolic energy. However, she was unable to observe any fusion of granules with the vacuoles formed by the invagination of the plasma membrane, although this has been observed repeatedly in other cells. In contrast to the route of entry of large particles, Zucker-Franklin (482) observed that small particles (such as cationized ferritin) appeared to enter the open canalicular system through a process of membrane flow that is not dependent on metabolic energy. Earlier investigators had come to a similar conclusion regarding the uptake of small particles.(492–494)

Interactions of Platelets with Bacteria

Many investigators have demonstrated that platelets adhere to a wide variety of bacteria and interact with them, but only a few have claimed that platelets actually phagocytose bacteria in a manner similar to phagocytosis by leukocytes. Many kinds of bacteria (both gram-positive and gram-negative) have been shown to induce platelet aggregation and cause the release of platelet granule contents. As pointed out by Herzberg and colleagues (488) interactions between platelets and bacteria are likely involved in septicemia, disseminated intravascular coagulation, and bacterial endocarditis. Studies with ^{111}In-labeled platelets show that platelets localize at sites of bacterial infection induced in experimental animals.(495) Clawson and White have shown by electron microscopy that platelets bind irreversibly to some bacteria (for example, *Staphylococcus aureus*) and as a result, the platelets aggregate and release granule contents.(486, 487) Adhesion requires divalent cations and the platelet changes are similar to those that occur when platelets adhere to collagen.(487) The bacteria are trapped within the platelet aggregates, but they are seldom seen within the platelets themselves.(487, 496) Although platelet interaction with bacteria can occur to some extent in the absence of plasma,(406, 496) it is potentiated by plasma proteins such as fibrinogen,(406, 496–498) immunoglobulin G,(499, 500) or components of the complement system.(501) Herzberg and associates (488) showed that the adhesion step of the interaction of platelets with *Streptococcus sanguis* was mediated by protease-sensitive components on the surfaces of the streptococci and of the platelets, involving surface microfibrils on *Streptococcus sanguis*, and that Ca^{2+} was not required.

With some bacteria, it may be the antigen–antibody complex, formed between the bacteria and antibodies in plasma, with which the platelets interact.(499) Thrombocytopenia is a frequent complication of bacterial infections, and Zimmerman and colleagues have suggested that the reaction among organisms such as pneumococci, antibodies present in most normal plasmas, and platelets, may be responsible for thrombocytopenia accompanying infections with such microorganisms.(499)

Endotoxin also interacts with platelets, but this aspect of platelet adhesion is beyond the scope of this review.

Reaction with Antigen–Antibody Complexes

The literature concerned with the reaction of platelets with antigen–antibody complexes is too extensive to be reviewed here. There is no doubt that platelets adhere to antigen–antibody complexes and are activated by them. In some species (e.g., man) adherence is through Fc receptors on the platelet membrane. In species that lack Fc receptors (e.g., rabbit), adherence appears to be mediated through C3 of the complement system.(502)

PHAGOCYTOSIS OF PLATELETS

Platelets are phagocytosed by polymorphonuclear leukocytes and monocytes in citrated plasma,(475) and by polymorphonuclear leukocytes and macrophages in thrombi.(183, 503, 504) As pointed out previously,(471) it may be only platelets that have

been altered by ingesting particulate matter, or by other stimuli, that can subsequently be removed by other phagocytic cells. Under some circumstances, opsonization of platelets by antiplatelet antibodies has been found to promote phagocytosis of platelets by leukocytes.(505, 506) This has been shown in vitro, but it may be involved in vivo in idiopathic thrombocytopenic purpura (ITP), in which antiplatelet antibodies are present and may promote the clearance of the sensitized platelets by the liver and spleen. Corticosteroids interfere with phagocytosis of platelets in ITP,(507) possibly by interfering with the initial adhesion reaction.(508)

The ability of macrophages to phagocytose platelets has been used as a means of delivering vinblastine-loaded platelets to these cells to destroy them. This technique has been reported to be successful in some cases of immune thrombocytopenia or autoimmune hemolytic anemia by Ahn and colleagues,(509, 510) although other investigators have found it to be beneficial in only an occasional patient with immune thrombocytopenia.(511) Panasci and coworkers have used vinblastine-loaded platelets to treat patients with thrombocytopenia associated with tumors that phagocytose platelets.(512)

Removal of Platelets from the Circulation

When it is time for a platelet to die, it undoubtedly adheres to phagocytic constitutents of the reticulo-endothelial system that are responsible for its removal from the circulation. This process is not well understood, but, by analogy with the removal of desialylated red blood cells, it may be carried out by the Kupffer cells of the liver and by mononuclear spleen cells.(513) It is known that platelets that have been treated with neuraminidase to remove surface sialic acid, or with proteolytic enzymes that remove glycopeptides from membrane glycoproteins, are rapidly cleared from the circulation.(432, 514) It is not known whether loss of these membrane components is responsible for removal of platelets at the end of their life span. It may be that the shortened platelet survival associated with continuous vessel injury is a result of the action of proteolytic enzymes on platelets, since administration of epsilon aminocaproic acid, an inhibitor of proteolytic enzymes such as plasmin, prolongs the shortened platelet survival caused by continuous vessel injury.(515)

Platelet survival is shortened in idiopathic immune thrombocytopenia and the amount of IgG associated with the platelets is greater than normal.(516) Adhesion of platelets to phagocytic cells is enhanced when IgG is bound to the platelets.(517) It has been suggested that if old platelets were altered in such a way that they could bind more IgG than young platelets, this opsonization might play a part in the clearance of the older platelets by macrophage phagocytois.(517, 518)

ABNORMALITIES RESPONSIBLE FOR DECREASED ADHESION

Several congenital abnormalities effect platelet adhesion to collagen or the subendothelium. At high shear rates, lack of plasma von Willebrand Factor diminishes platelet adhesion. Platelets from patients with the Bernard Soulier syndrome lack glycoprotein Ib and fail to bind von Willebrand Factor in the presence of ristocetin. Their ability to adhere to the subendothelium at high shear rates is also impaired. Antibodies to glycoprotein I inhibit platelet adhesion to the subendothelium (361) and to collagenase-treated subendothelium.(367) Both of these abnormalities are discussed in detail elsewhere in this chapter (see section on *Binding of von Willebrand Factor to Platelets).*

In Glanzmann's thrombasthenia, platelet adhesion to the subendothelium was shown to be normal, as tested in the Baumgartner perfusion chamber.(320, 369) As would be expected, no platelet aggregates formed. Baumgartner and associates (320) also investigated the adhesion of thrombasthenic platelets to the fibrillar collagen of the subendothelium after it had been digested with chymotrypsin. They observed that platelet adhesion (contact plus spread platelets) tended to be low, but they suggested that low platelet counts and a low hematocrit may have been responsible. Surface coverage with "contact" platelets was higher than in controls and their detailed morphologic study showed that fewer platelets were spread on the surface, although the "contact" platelets had lost their granule contents. These observations were interpreted as an indication that thrombasthenic platelets do not move along the collagen fibrils to the solid surface of the internal elastic lamina as normal platelets do.(320) It is evident, however, that glycoproteins IIb and IIIa, which are missing from thrombasthenic platelets, do not have a significant role in platelet adhesion to the subendothelium or to collagen.

In storage-pool disease (deficiency of dense granules) platelet adhesion was found to be decreased by about 40 percent in 4 of the 6 patients studied by Weiss and colleagues.(135) Although large thrombi did not form, some small thrombi were observed. Adhesion to the collagen fibrils of chymotrypsin-treated subendothelium was also impaired in 3 of 6 patients tested.(320) The investigators concluded that these storage pool-deficient platelets had a defect in their ability to spread on the surface. Similar observation were obtained with storage pool-deficient rat platelets.(519)

Probably because individuals with the gray platelet syndrome (α-granule deficiency) are extremely rare, no reports have appeared concerning the ability of these platelets to adhere to the subendothelium.

In the single patient with a variant of Ehlers-Danlos syndrome in which fibronectin corrected the defect in collagen-induced platelet aggregation,(405) no attempt was made to measure platelet adhesion. As mentioned earlier, aggregation responses to other aggregating agents were also abnormal so the nature of the defect is not entirely clear.

INHIBITORS OF PLATELET ADHESION

Many inhibitors of platelet aggregation have been examined for their effects on platelet adhesion. These studies have had at least two aims: to determine whether drugs can be used to inhibit the first event in thrombus formation on injured blood vessel, and to investigate the biochemical reactions involved in platelet adhesion.

Inhibitors with Structural Similarities to Collagen

The peptide fragments of collagen that block the interaction of platelets with collagen have been discussed earlier (235, 241) and the inhibitory effect of poly-L-hydroxyproline has been mentioned.(145, 229) Another molecule that is structurally similar to part of the collagen molecule is C1q, a subcomponent of the first component (C1) of the complement system.(520, 521) Several investigators have shown that C1q inhibits collagen-induced platelet aggregation.(522–526) Platelet adhesion to collagen (types I and III) is also inhibited.(522, 525) It appears that the portion of C1q responsible for these effects is the A chain of C1q, which contains collagen-like amino acid sequences.(525) In contrast to monomeric C1q, aggregated C1q behaves like collagen and induces the release of the

contents of platelet granules.(522) It seems probable that C1q becomes associated with the sites on the platelet membrane that normally interact with collagen and hence, prevents platelet adhesion to collagen and the subsequent reactions.

C1s (another subcomponent of C1) has also been shown to inhibit platelet adhesion to types I and III collagen, and collagen-induced platelet aggregation.(527) Since C1s binds to C1q through the collagen-like moiety of the latter, it has been suggested that C1s exerts its inhibitory effect by binding to collagen and preventing platelet adhesion by blocking the sites on the collagen molecule to which platelets adhere.

Inhibition by Chelation of Divalent Cations

The inhibitory effect of chelation of divalent cations on platelet adhesion to collagen or the subendothelium has already been described (see *Role of* Ca^{2+} and Mg^{2+} in Platelet Adhesion).

Inhibition by Sulfhydryl Inhibitors

Some years ago, Al-Mondhiry and Spaet (528) showed that the penetrating sulfhydryl inhibitors N-ethyl maleimide (NEM) and p-hydroxymercuribenzoate (PCMB) immediately blocked the ability of platelets in EDTA-platelet-rich plasma to adhere to connective tissue, whereas the more slowly penetrating p-hydroxymercuriphenosulfonate (PCMBS) was inhibitory only after prolonged incubation. Cazenave and associates (143) also observed inhibition by NEM of platelet adhesion to a collagen-coated surface with platelets in a calcium-containing suspending medium, but could not demonstrate inhibition by PCMB at concentrations that did not cause release of granule contents and lysis. Al-Mondhiry and Spaet (528) had suggested that the sulfhydryl group inhibitors affected internal platelet reactions, and Cazenave and colleagues (143) implicated the platelet contractile protein because other inhibitors of platelet shape change and clot retraction also diminished platelet adhesion. These included prostaglandin E_1, caffeine, adenosine, cytochalasin B, and colchicine.

Inhibition by Nonsteroidal Antiinflammatory Drugs

There has been much interest in the effects of nonsteroidal antiinflammatory drugs, particularly aspirin, on platelet adhesion, and contradictory reports have appeared.

Inhibition of adhesion by aspirin was reported by Jamieson and colleagues,(215) using the technique of Spaet and Lejnieks,(281) although these investigators had found no effect of aspirin on platelet adhesion in EDTA-platelet-rich plasma. Others who have observed inhibition by aspirin in vitro include Sheppard,(529) Cowan,(530) and Cazenave in early experiments (see below). Reports of no inhibition of platelet adhesion in vitro by aspirin have come from MacKenzie,(279) MacIntyre and Gordon,(531) Legrand and colleagues,(139) Morin and coworkers,(359) Weiss and Tschopp,(135, 136) Tschopp,(136) Cazenave and coworkers,(137, 532) and Davies and colleagues.(533) In vivo, aspirin does not appear to inhibit adhesion to the subendothelium (6, 529, 534) although it inhibits aggregate formation on the adherent platelets.

In early experiments, Cazenave and colleagues (535, 536) observed that aspirin inhibited the adhesion of washed platelets to a collagen-coated glass surface or the subendothelium, but in later studies using the rotating probe technique and a hematocrit of

40 percent, no effect of aspirin (100 μM) on adhesion of ^{51}Cr-labeled rabbit platelets was demonstrable.(532, 533) In contrast, 100 μM indomethacin inhibited adhesion to collagen-coated glass by about 30 percent in this system, but similar inhibition by sulfinpyrazone was evident only at 1 mM, which is too high to be relevant to the in vivo situation.(137) The later studies with a 40 percent hematocrit in the platelet suspending medium were done in media containing 2 mM Ca^{2+} and 1 mM Mg^{2+}, but the surfaces were rinsed in solutions containing EDTA after adherence had taken place. This technique removes any rabbit platelets that have aggregated on the adherent platelets, and therefore measures only platelet adhesion. In the earlier experiments, the surfaces were rinsed repeatedly in modified Tyrode solution without added Ca^{2+} or Mg^{2+}, and apyrase was present to remove released ADP, but these experiments have been criticized on the grounds that a few aggregates may have remained on the surfaces (320); the formation of aggregates would be inhibited by aspirin and therefore it would appear that aspirin had inhibited adhesion.

In nearly all of the reports by Baumgartner, Weiss, and their colleagues, with citrated or native whole blood, aspirin has not been found to inhibit platelet adhesion to the subendothelium. They did find, however, that at high shear rates, aspirin was slightly inhibitory, although no effect was observed with native blood.(138) Thus there now seems to be general agreement that platelet adherence to collagen or the subendothelium is not appreciably inhibited by aspirin in vivo, or in vitro in whole blood, or in platelet suspensions containing red blood cells at a hematocrit of 40 percent.* It is evident that conditions of flow, hematocrit, and anticoagulant affect the results of experiments in which the techniques used have varied widely. Even when precautions have been taken to avoid platelet aggregation on the adherent platelets, consistent observations have not been obtained. It does not seem likely, however, that aspirin has a large inhibitory effect on platelet adhesion to damaged vessel walls in vivo.*

As mentioned earlier, the nonsteroidal antiinflammatory drugs do not inhibit the release of granule contents from adherent platelets.(97, 135, 137) Thus it is not surprising that aspirin has not been found to inhibit the smooth muscle cell proliferation that follows removal of the endothelium.(24, 537)

Other nonsteroidal antiinflammatory drugs that have been found to inhibit platelet adhesion under some conditions are sulfinpyrazone and indomethacin.(137)

Inhibition of Adhesion by Prostaglandins that Increase Platelet cAMP

Inhibition of platelet adhesion in vitro by PGE_1 was demonstrated some time ago by Cazenave and colleagues (143, 434) and by Baumgartner and coworkers,(33) and attributed to its ability to increase the concentration of cyclic AMP in platelets. The potential importance of agents that raise the concentration of cyclic AMP in platelets did not become apparent until the discovery of PGI_2 (prostacyclin). This compound is produced by the endothelium upon stimulation and it not only inhibits platelet aggregation and the release of the contents of platelet granules, but it inhibits platelet adhesion to collagen and the subendothelium.(538–542) The concentration required to inhibit adherence is higher than that required to inhibit aggregation, however, and only approximately 50 percent inhibition was achieved at concentrations as high as 10 μM.(538) Weiss and Turitto (540)

*References 97, 134–138, 280, 532, and 533.

were also unable to inhibit platelet adhesion to the subendothelium completely at any of the concentrations they tested. Stable analogs of prostacyclin also inhibit adhesion.(541, 543) Although high concentrations of PGE_1 or PGI_2 inhibited the release of granule contents from adherent rabbit platelets, they did not affect human platelets in this way.(538) Thus PGI_2 may decrease platelet adhesion at an injury site, but it may not inhibit the release of platelet-derived growth factor from the platelets that do adhere. Any effect of PGI_2 in vivo is likely to be a local effect because the concentration of circulating PGI_2 is now known to be too low to affect either platelet adhesion or aggregation.(544)

Karniguian and coworkers (542) observed inhibition of adhesion of human platelets by PGI_2, PGD_2, PGE_1, and dibutyryl cyclic AMP. They also reported inhibition of release, but in the system they used this may have been inhibition of release from platelets that were not adherent to collagen.(139)

The thromboxane synthetase inhibitor, dazoxiben, has been shown to inhibit platelet adhesion to the subendothelium of the rabbit aorta.(148) It was suggested that prostaglandin endoperoxides liberated from platelets in the presence of this inhibitor contributed to PGI_2 formation by the vessel wall, and that this PGI_2 was responsible for the inhibitory effect on adhesion.

In experiments in which PGI_2 was infused into rabbits, Adelman and colleagues (21) showed prevention of platelet adhesion to the subendothelium of rabbit aortas from which the endothelium had been removed with a balloon catheter. At an infusion rate of 650 to 850 ng/kg/min, surface coverage by platelets was reduced by 84 percent and attachment by 63 percent. They also found that secretion of platelet factor 4 into the underlying vessel wall was prevented by the infusion. These observations with rabbit platelets in vivo are in agreement with the in vitro results of Cazenave and colleagues (538) with rabbit platelets.

It should be emphasized, however, that PGI_2 is unlikely to be responsible for the nonthrombogenic nature of the vascular endothelium, or for the development of a nonthrombogenic surface after vascular injury, because inhibition of PGI_2 formation with aspirin in vitro or in vivo does not promote platelet adhesion to those surfaces.(6) In addition, aspirin or indomethacin does not increase the adherence of platelets to altered endothelial cells in tissue culture.(74, 76)

An additional consideration concerning the role of endogenously produced PGI_2 in limiting platelet adhesion to damaged vessel walls is the well established observation that the cells in the vessel wall underlying the endothelium have much less ability to form PGI_2 than the endothelial cells.(545–548) It may be that the endothelial cells surrounding an injury site where the endothelium has been lost are mainly responsible for the production of any PGI_2 that limits thrombus formation.

When the neointima that forms on a vessel wall after an initial injury is damaged, platelets adhere to collagen, and also to fibrin at the site.(18, 38) Thus it is not surprising that the combination of PGI_2 and heparin is more effective than either agent alone in limiting platelet accumulation on the injured neointima.(39)

Effect of Dipyridamole on Platelet Adhesion in Vitro

Platelet adherence to the subendothelium of the rabbit aorta, to collagen, or to a collagen-coated glass surface, is inhibited by high concentrations of dipyridamole.*

*References 33, 173, 359, 434, 535, and 549.

Although dipyridamole is a cAMP phosphodiesterase inhibitor that can increase the concentration of cAMP in platelets when adenylate cyclase has been stimulated by agents such as PGE_1 or PGI_2, it is not clear that this is its mode of action in inhibiting platelet adhesion because, by itself, it has no detectable effect on the concentration of cAMP in platelets.(550)

Baumgartner and colleagues (33) showed that dipyridamole (1 m*M*) inhibits the initial attachment of platelets to the subendothelium by about 50 percent. Approximately 50 percent inhibition by 100 μM dipyridamole, but little or no inhibition by 10 μM was observed by Groves and colleagues (549) with a rotating probe system in which the adherence of ^{51}Cr-labeled platelets to the subendothelium was assessed; the platelets had been washed and resuspended in Eagle's medium containing 4 percent albumin and red blood cells at 40 percent hematocrit. At a concentration of 100 μM, dipyridamole also partially inhibited the release of ^{14}C-serotonin from the adherent platelets, and reduced the extent of collagen-induced platelet aggregation to 66 percent of the control value.

In Vivo and Ex Vivo

In experiments with rabbits in which the endothelium of the aorta was removed with a balloon catheter, Groves and colleagues (549) found that dipyridamole (12.5 or 2.5 mg/kg), given intravenously 10 minutes before removal of the endothelium, significantly reduced the number of platelets that accumulated on the injured surface during the 10 minute period immediately following exposure of the subendothelium. The plasma concentration of dipyridamole 15 minutes after administration of the higher dose was 27 μM. The effect of dipyridamole on adherence appeared to be a consequence of the action of dipyridamole alone, since inhibition of PGI_2 formation with aspirin did not influence the effect of dipyridamole. These observations indicate that PGI_2 formed by injured vessels does not potentiate inhibition of adhesion by dipyridamole.

In contrast to the in vivo findings of Groves and colleagues, Weiss and coworker (138) could not demonstrate an effect of dipyridamole on adhesion, but the experimental design was quite different. In their studies, human subjects ingested 150 mg of dipyridamole and then continued to take the drug (100 mg q.i.d.) for 6 days. Tests of platelet adhesion were done before the first dose, 1.5 hours after the first dose and 1.5 hours after the last dose. Citrated or native blood was circulated through an annular Baumgartner chamber on whose inner core were mounted everted segments of de-endothelialized rabbit aorta. The wall shear rate was 2600 sec^{-1}. Adherent platelets, and platelet thrombi, were assessed morphometrically. Total serum dipyridamole concentrations were 2.6 and 3.6 μM, at 1.5 hours and 6 days, respectively. The lack of effect of dipyridamole in these experiments is probably attributable to the low concentration in the plasma, although species differences and the ex vivo system with its high shear rate may also contribute to the discordance with the results obtained in vivo by Groves and colleagues.(549) It is also difficult to understand the results of McCollum and coworkers (551) on platelet adhesion to Dacron vascular grafts in an ex vivo system. Human blood from volunteers who had ingested 100 mg q.i.d. of dipyridamole for 1 week was collected into heparin and pumped through circuits containing preclotted woven Dacron. The effect of the drug on the fall in platelet count during passage through the graft material was determined. The plasma concentration of dipyridamole was 2.7 μM. In this system, dipyridamole reduced platelet adherence, but it was less effective than aspirin. The surface of the Dacron prosthesis, however, differs greatly from the subendothelium, so comparisons of the effects of drugs may not be valid. Nevertheless, it is apparent that under some conditions, both in vitro

and in vivo, dipyridamole can partially inhibit platelet adhesion to the subendothelium, collagen, or Dacron grafts, particularly when the drug is used at high concentrations.

Clinical Effects of Dipyridamole

It is tempting to speculate that at least part of the beneficial effect of dipyridamole observed by the group at the Mayo Clinic may be attributable to inhibition of platelet adhesion and release at sites of vascular injury. They observed that administration of dipyridamole before and after surgery combined with aspirin after surgery, reduced the amount of intimal thickening that developed after the insertion of coronary bypass vein grafts in dogs,(552) and reduced the deposition of ^{111}indium-labeled platelets.(553) A reduction in the number of adherent platelets would diminish the amount of platelet-derived growth factor that entered the vessel wall and thus lessen intimal thickening. In similar experiments, Josa and associates (554) observed that this drug treatment reduced the incidence of early thrombosis on this type of graft in dogs. In endarterectomized carotid arteries from dogs, thrombus formation was also decreased by this treatment.(555) In a clinical trial, the group at the Mayo Clinic administered dipyridamole preoperatively, and the combination of dipyridamole and aspirin postoperatively. This treatment reduced the incidence of occlusion of coronary artery bypass grafts.(556, 557)

Other Inhibitory Drugs

Other drugs that inhibit platelet adhesion in vitro under physiologic conditions of divalent cation and protein concentrations, hematocrit, and blood flow, include methyl-prednisolone, penicillin G, and cephalothin,(137) but in most cases the concentrations required are higher than those likely to be achieved in vivo in man. Platelets from subjects receiving high doses of α-tocopherol adhere less readily to collagen in a system containing EDTA.(558)

VESSEL WALL INJURY

Platelets do not adhere to the vessel wall unless it has been injured in some way. Many methods have been devised to injure vessels in experimental animals for the purpose of studying platelet adhesion and thrombus formation. These will not be discussed in detail here. They include passage of a balloon-catheter through large blood vessels, placement of indwelling catheters in the aorta, air-drying, chemical, electrical, or laser injury, infusion of homocysteine, administration of diets enriched with cholesterol, and many others.

Most interesting are the naturally-occurring conditions that cause repeated damage of vessel walls and may be responsible for platelet adhesion to the wall and the contribution of platelets to the development of atherosclerosis. This subject has been reviewed recently.(559, 560) Repeated injury of vessel walls, regardless of how it is caused, has been shown to shorten platelet survival, undoubtedly as a result of changes in platelets that have adhered to the injury sites and then been freed to recirculate. Some injury may be caused by hemodynamic forces at vessel branches, curves, and stenoses.(561) Vessel wall injury and shortened platelet survival are associated with smoking (562, 563); although the injurious substance has not been identified, suggestions include carbon monoxide, nicotine, and tobacco antigen.(564–566) Diets rich in cholesterol have been

shown to cause vessel injury, platelet adherence, and shortened platelet survival in monkeys,(567, 568) and diets rich in animal fats shorten platelet survival in man.(569) Endothelial injury and shortened platelet survival have been described in homocystinemia by some investigators,(570, 571) although others have not detected shortened platelet survival.(572, 573) Vessel wall injury and early development of atherosclerosis and its clinical complications is a feature of diabetes; shortened platelet survival and platelet hypersensitivity have been reported in some diabetics.(574–577) Some arterial wall injury may result from immunologic reactions; serum sickness and systemic lupus erythematosus may be examples of this.(578–580) Viruses may also contribute to vessel wall injury.(581)

The reason for shortened platelet survival in association with vessel wall injury and platelet adhesion is not established. It may be that platelets that have adhered to the surface of an injured vessel can be freed by proteolysis of their membrane proteins or glycoproteins that are involved in adhesion. Enzymes that might be responsible for the cleavage of membrane glycoproteins are plasmin, leukocyte elastase, and the proteases associated with the subendothelium.(28–31, 368, 514, 582) Several lines of evidence support this theory: cleavage of glycopeptides from platelet membrane glycoproteins by treatment with plasmin, chymotrypsin, or trypsin shortens the survival of the platelets when they are returned to the circulation (514); epsilon amino caproic acid, an inhibitor of plasmin and other proteases, prolongs shortened platelet survival caused by continuous vessel injury (515); Bernard Soulier platelets, which lack glycoprotein Ib, show an abnormally short platelet survival time.(583, 584) It is not known whether the ability of dipyridamole to inhibit platelet adhesion (33, 549) is related to the observation that this drug prolongs shortened platelet survival.(585)

SUMMARY

Platelets do not adhere to surfaces to which flowing blood is normally exposed in vivo. When the lining of a blood vessel is altered or damaged, however, platelets do adhere to the injured site. Platelet adhesion is one of the first events in the formation of hemostatic plugs and thrombi, and plays a part in the development of atherosclerotic lesions. Other surfaces to which platelets adhere include particulate matter in the blood stream, bacteria and other microorganisms, the artificial surfaces of prosthetic devices, and some altered cells in the blood, particularly macrophages. The majority of investigators have studied the interaction of platelets with the subendothelium of normal vessels of young animals, or with isolated vessel wall constituents such as collagen. There are very few studies of platelet adhesion to repeatedly damaged or diseased blood vessels, although it is generally assumed that platelets interact with the connective tissue, fibrin, and cholesterol crystals in atherosclerotic lesions.

Underlying the endothelium of blood vessel is the basement membrane, which has been shown to contain type IV collagen, elastin with its associated microfibrils, von Willebrand Factor, fibronectin, thrombospondin, laminin, and heparan sulfate. If only the endothelium is removed, the main structure exposed is the basement membrane with its associated proteins, but deeper injuries expose fibrillar type III collagen and microfibrils. In most studies in which large arteries have been injured by passage of a balloon catheter, basement membrane, type III collagen and the microfibrils around elastin have been exposed. Platelets do not react strongly with basement membrane and the type IV colla-

gen in it is relatively inert. In contrast, platelets adhere firmly to type III (and type I) collagen and spread on it. Although in vitro studies have shown that platelets can interact with collagen in artificial media without plasma proteins, investigations of platelet adhesion at high shear rates indicate that von Willebrand Factor is necessary for firm platelet adhesion under these conditions. Fibronectin and thrombospondin may also have a role in platelet adhesion. However, platelets do not bind von Willebrand Factor or fibronectin until the platelets have been stimulated to release their granule contents, so these binding sites probably do not become available until the platelets have interacted with collagen or another release-inducing agent such as thrombin. Studies with platelets from patients with the Bernard Soulier syndrome in which glycoprotein Ib on the platelet membrane is missing have demonstrated that this glycoprotein takes part in platelet adhesion at high shear rates.

Type I and type III collagen have been studied extensively to determine the characteristics required for platelet adhesion and the subsequent release of granule contents, formation of products such as thromboxane A_2 from arachidonate freed from membrane phospholipids, and aggregation. To induce platelet adhesion and the resulting platelet reactions, triple helical collagen must be assembled into a quaternary structure that is described as fibrillar or multimeric. The epsilon amino groups of lysine on collagen are of major importance in binding to platelets, whereas the telopeptide regions and the carbohydrate side chains have little or no effect. Some investigators have isolated peptide fragments of collagen that interact with platelets, and others have advanced the theory that there are multiple, simultaneous, linked interactions between platelets and collagen. No receptor for collagen has been identified on the platelet surface. A number of techniques have been used to quantify platelet adhesion to collagen or the subendothelium under a variety of conditions of suspending medium, flow, hematocrit, and divalent cation concentration. All of these variables affect the extent of platelet adhesion. The hemodynamic forces of blood flow, including the effect of red blood cells, determine the rate of adhesion of platelets to a surface. When blood flow is disturbed, extensive aggregation can occur on the adherent platelets, with fibrin formation around the aggregates.

When small blood vessels are cut, the exposed collagen provides a strong stimulus for platelet adhesion and aggregation. This process is enhanced by the high shear rates in the microcirculation. Under these conditions, a deficiency of von Willebrand Factor leads to decreased adhesion of platelets and less spreading on the injured surface. This is undoubtedly the reason for the hemostatic defect in von Willebrand's disease. In medium to large arteries where the shear rates are lower, the defect is not apparent. There have been many studies of the binding of von Willebrand Factor to platelets and its role in platelet adhesion. Recent evidence indicates that von Willebrand Factor may be involved in the reaction of platelets with the microfibrils around elastin as well as with collagen.

Platelets adhere to fibrin in hemostatic plugs and thrombi; this reaction is involved in clot retraction and the consolidation of hemostatic plugs.

The exposed subendothelium remains reactive to circulating platelets for only a short time. When it becomes covered with platelets, the surface of the adherent platelets does not attract circulating platelets under conditions of laminar flow. After several days, platelets are no longer apparent on the surface and it is relatively inert.

The neointima that forms in large arteries after removal of the endothelium is composed of smooth muscle cells that present a nonthrombogenic surface to the blood. When these cells are damaged, thrombi composed of platelets and fibrin form on the surface, and fibrin is often apparent between the platelets and the injured surface. Thrombin plays

a major role in the formation of these thrombi by affecting both fibrinogen and platelets. Platelet adhesion to fibrin as well as to collagen occurs on such a surface. As with the exposed subendothelium, however, the surface rapidly becomes nonreactive to further platelet deposition.

Many drugs have been examined for their effects on platelet adhesion, since it would be beneficial if this first reaction in thrombus formation could be inhibited. Drugs that inhibit the change in the shape of platelets that occurs upon stimulation have been shown to inhibit adhesion. The most effective drugs that act in this way are those that increase the concentration of cAMP in platelets (PGE_1, PGI_2, dipyridamole). The nonsteroidal antiinflammatory drugs that inhibit the formation of thromboxane A_2 do not affect platelet adhesion under conditions similar to those in vivo, nor do they prevent the release of granule contents from the adherent platelets. When thrombin and fibrin, as well as collagen, are involved in platelet adhesion and thrombus formation, a combination of heparin with PGE_1 or PGI_2 is more inhibitory than either agent by itself.

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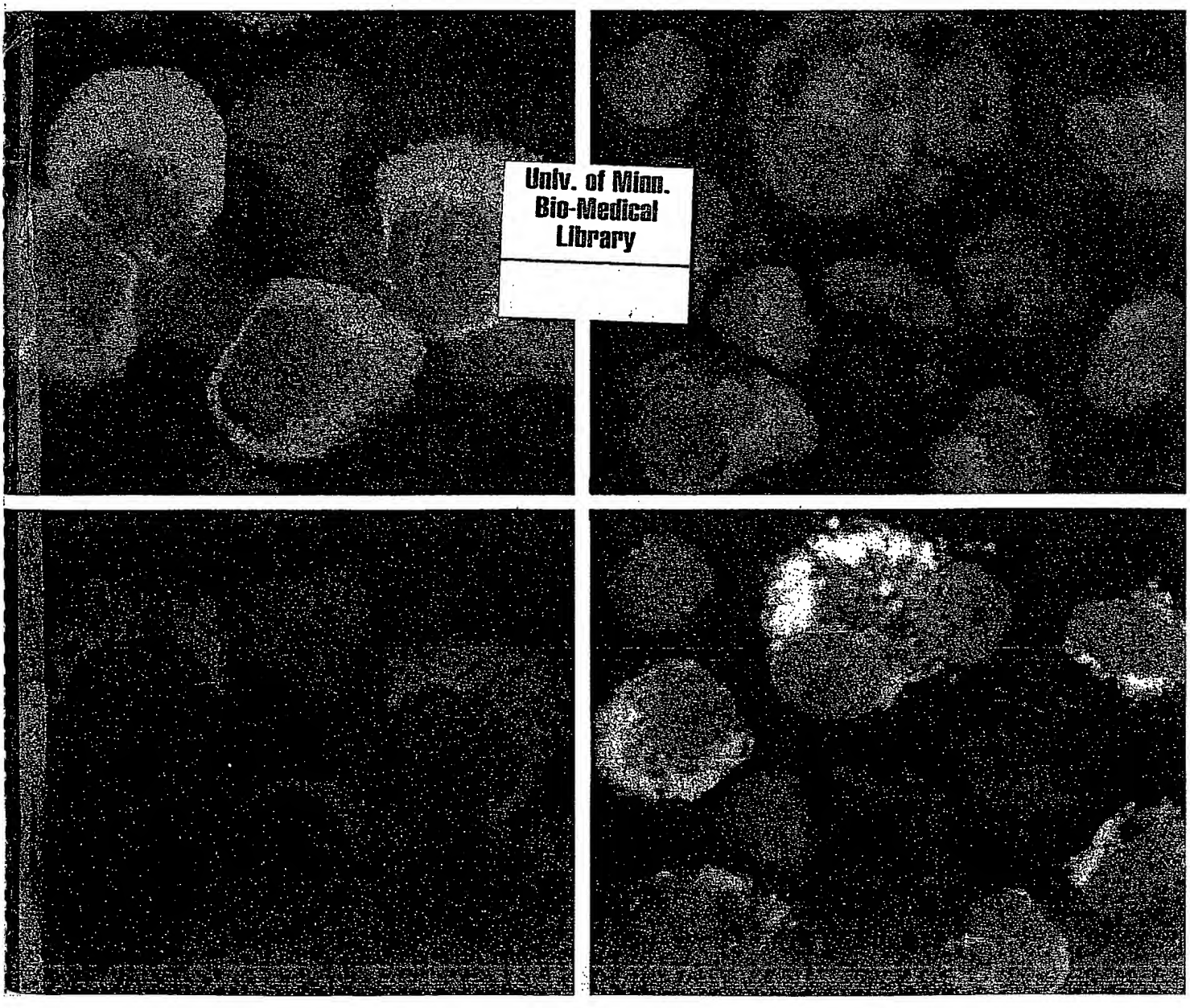

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Flow cytometric analysis of platelet activation by different collagen types present in the vessel wall

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Summary. The interaction of platelets with collagens of the vessel wall is a critical event in primary haemostasis. Although numerous studies have examined the ability of various collagen types to support platelet adhesion, little is known concerning the relative ability of different collagens to elicit specific activation markers in platelets. In this report, flow cytometric analysis has been utilized to evaluate the ability of various native collagen types to elicit secondary activation events in human platelets. Collagen types I, III, V and VI induced α-granule secretion and up-regulation of cell surface glycoprotein (GP) IIb/IIIa. In contrast, collagen type IV did not elicit these responses in the concentration ranges examined. Dose–response curves for α-granule secretion induced by the various collagen types indicated that human type III and human type I collagens were less effective than human type V, human type VI and calf skin type I. In addition, the ability of these various collagens to activate GPIIb/IIIa to its ligand binding conformation was even more heterogenous with only human type VI and calf skin type I readily promoting this transition. These data demonstrate that flow cytometric analysis of collagen-induced platelet activation is feasible and that collagen-mediated α-granule secretion and membrane glycoprotein redistribution in human platelets are separate events from activation of GPIIb/IIIa.

Keywords: platelet activation, collagen, P-selectin, glycoprotein IIb/IIIa, flow cytometry.

Platelet adherence to collagen is recognized as a critical initial event for generation of a haemostatic plug (Sixma *et al*, 1997). The complexity of this interaction is emphasized by the presence of at least seven different forms of collagen within the vessel wall (van der Rest & Garrone, 1991) and reports of several different collagen receptors on platelets (Moroi & Jung, 1997). To dissect the multitude of possible interactions, investigators have examined platelet binding to various forms of immobilized collagen under conditions of both flow and stasis (for reviews see Sixma *et al*, 1997; Moroi & Jung, 1997; Kehrel, 1995). These studies suggest that most fibrillar forms of collagen will support adhesion, activation and aggregation of platelets to varying degrees (Moroi & Jung, 1997).

The primary collagen receptor on platelets is considered by many to be GPIaIIa (integrin $\alpha_2\beta_1$) which is present on the platelet surface at ~1000–2000 copies/cell (Saelman *et al*, 1994; Kunicki *et al*, 1993). However, there are data to suggest that GPIV (CD36) (Tandon *et al*, 1989), GPVI (Kehrel *et al*, 1998) and a 65 kD membrane protein (Chiang *et al*, 1997) are also involved with collagen binding. The data for each receptor include antibody inhibition studies, direct receptor isolation and/or absence of collagen-induced responses in patients lacking specific membrane receptors. Each receptor is assumed to be constitutively active although some potentiation of receptor affinity has been reported (Wilkins *et al*, 1996), and several investigators have suggested that full activation of platelets requires synergistic cooperation between different forms of collagen receptors (Keely & Parise, 1996; Clemetson, 1995).

The examination of platelet function has been greatly facilitated by the introduction of flow cytometric techniques which allow analysis of adhesive protein binding (Jackson & Jennings, 1989; Heilmann *et al*, 1994) and secondary events elicited by activation (Johnston *et al*, 1987; Michelson *et al*, 1994; Michelson, 1992). Particularly relevant among these secondary activation events are the redistributions of membrane glycoproteins (Nurden, 1997) and activation of specific receptors (Ginsberg *et al*, 1995). However, the collagen–platelet interaction has not been considered amenable to flow cytometric study due to the polymerization of collagen in neutral solution resulting in the formation of insoluble macro-aggregates (Williams *et al*, 1978). These aggregates are large enough to interfere with light-scatter

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analysis during flow cytometry (Wierwille *et al*, 1997) as well as to simultaneously interact with multiple platelets. One approach to this problem utilized by other investigators employs methylated collagens which are slow to aggregate and therefore can be used for flow cytometric analysis of the platelet–collagen interaction (Wierwille *et al*, 1997).

In this report we present a flow cytometric study of activation events in platelets induced by native collagens; this was accomplished by utilizing low concentrations of unmodified collagens under experimental conditions which minimized collagen aggregation. This methodology allows an examination of the ability of various collagens to elicit α-granule secretion, glycoprotein redistribution and glycoprotein receptor activation in human platelets. These data demonstrate that several collagen types mimic traditional strong agonists whereas others do not.

MATERIALS AND METHODS

Materials. Collagens type I (calf skin), type I (human), type III (human), type IV (human), type V (human), Sepharose CL-2B, FITC-goat-anti-mouse-IgM (FITC-GAMM) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St Louis, Mo. Collagen type VI (human) was obtained from Heyltex Corp., Houston, Texas. Fluorescein isothiocyanate (FITC) and NHS-biotin were obtained from Calbiochem, La Jolla, Calif. Phycoerythrin-labelled streptavidin (PE-SA) was obtained from Molecular Probes, Eugene, Ore. Monoclonal antibody G5 recognizes human P-selectin and was provided by Dr R. McEver, Oklahoma Medical Research Foundation, Oklahoma City, Okla.; antibody AP2 against human GPIIb/IIIa (Pidard *et al*, 1983) was provided by Dr T. Kunicki, Scripps Research Institute, La Jolla, Calif.; antibody PAC-1 which recognizes activated GPIIb/IIIa (Shattil *et al*, 1987) was provided by Dr S. Shattil, Scripps Research Institute, La Jolla, Calif.

Buffers. BSGC: buffered saline-glucose-citrate, 129 mM NaCl, 13·6 mM Na_3citrate, 11·1 mM glucose, 1·6 mM KH_2PO_4, 8·6 mM NaH_2PO_4, pH adjusted with NaOH to either 6·5 or 7·3. ACD: acid citrate dextrose, 38·1 mM citric acid, 74·8 mM Na_3citrate, 136 mM glucose. PBS: phosphate buffered saline, 150 mM NaCl, 10 mM NaH_2PO_4, pH 7·4. Saline: 150 mM NaCl. HEPES: 100 mM HEPES, pH 7·5.

Human platelets. Informed consent was obtained in accordance with local Institution Review Board guidelines. 5 ml blood was drawn into 0·5 ml ACD and then diluted with 5 ml of room temperature BSGC, pH 7·3. Platelet-rich plasma (PRP) was prepared in 12×75 mm plastic centrifuge tubes filled maximally and centrifuged at 170 *g* for 8 min at room temperature. 2 ml of PRP were removed and applied to a 25×55 mm column of Sepharose CL-2B equilibrated with BSGC, pH 6·5. Isolated platelets were counted and diluted in BSGC, pH 7·3 to a cell count of 4×10^7/ml.

Collagen solutions. Collagens were dissolved at 1 mg/ml in 85 mM acetic acid overnight at 4°C. Stock solutions were prepared with a 1:5 dilution with water to yield a final collagen concentration of 200 μg/ml in 17 mM acetic acid; these optically clear stock solutions were stored at 4°C in glass tubes and were stable for at least a month. In one set of experiments calf skin collagen at 200 μg/ml in 17 mM acetic acid was centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant was then compared to the starting collagen solution for its ability to activate platelets utilizing the assays described below. Centrifugation did not affect the ability of collagen to mediate platelet activation, thereby indicating that there was no significant level of pre-formed collagen oligomers in these stock solutions. Further analysis of oligomerization after neutralization is discussed below.

Platelet activation markers. Binding of 5 μg/ml biotin-G5, an anti-P-selectin antibody, was used to monitor α-granule secretion by activated human platelets. GPIIb/IIIa redistribution was monitored with 5 μg/ml FITC-AP2 (Pidard *et al*, 1983). GPIIb/IIIa activation in human platelets was monitored with 5 μg/ml PAC-1 (Shattil *et al*, 1987) which was subsequently detected with FITC-GAMM.

Collagen activation of platelets. Reactions were performed in 17×100 mm polypropylene round-bottom culture tubes. For a final collagen concentration of 20 μg/ml, 50 μl of collagen (200 μg/ml stock) and 150 μl of 17 mM acetic acid in saline were added to reaction tubes and kept on ice until needed. Immediately before the assay was initiated, 250 μl of RT 100 mM HEPES, pH 7·5, with 2 mM $CaCl_2$ and any relevant antibody (e.g. G5 or PAC-1) was added followed by 50 μl of gel-filtered platelets. The reaction proceeded at 37°C for 10 min and was stopped with 4 ml of ice-cold 1% formalin in PBS. After 20 min of fixation at RT, 8 ml of 1 mg/ml BSA in PBS (BSA-PBS) were added, the platelets pelleted at 1500 *g* for 15 min, and the pellet resuspended in 400 μl BSA-PBS. 200 μl of the fixed platelets were transferred to 12×75 mm polypropylene tubes and labelled with the respective detection system, 5 μg/ml PE-SA for biotinylated antibodies, 10 μg/ml FITC-GAMM for PAC-1 or 5 μg/ml FITC-AP2 for GPIIb/IIIa. After 30 min of labelling at RT, the platelets were washed by addition of 3 ml of BSA-PBS and centrifuged at 1500 *g* for 15 min. The final pellet was resuspended in 800 μl of BSA-PBS for flow cytometric analysis.

Flow cytometry. Flow cytometric analysis was performed on a FACScan instrument (Becton Dickinson, San Jose, Calif.) utilizing ConSort 30 software. Platelets were identified by their characteristic forward scatter/side scatter or with platelet-specific antibodies (Pidard *et al*, 1983).

Flow cytometric analysis of collagen-induced changes was possible as a result of conditions which prevented any detectable collagen polymerization during the assay. These conditions included use of dilute stock solutions of collagen in weak acid, maintenance of low collagen concentrations after acid neutralization, and immediate utilization of collagen solutions upon neutralization as detailed above. With these experimental conditions, no collagen oligomers interfering with flow cytometric examination were observed utilizing forward light scatter/side light scatter analysis. Two additional controls were performed. First, collagen at 10 μg/ml was neutralized with HEPES buffer as described above and pre-incubated at 37°C for time periods ranging from 0 to 10 min before addition of platelets; no alteration in platelet response was observed. If collagen oligomerization upon neutralization were a significant contributor to the described

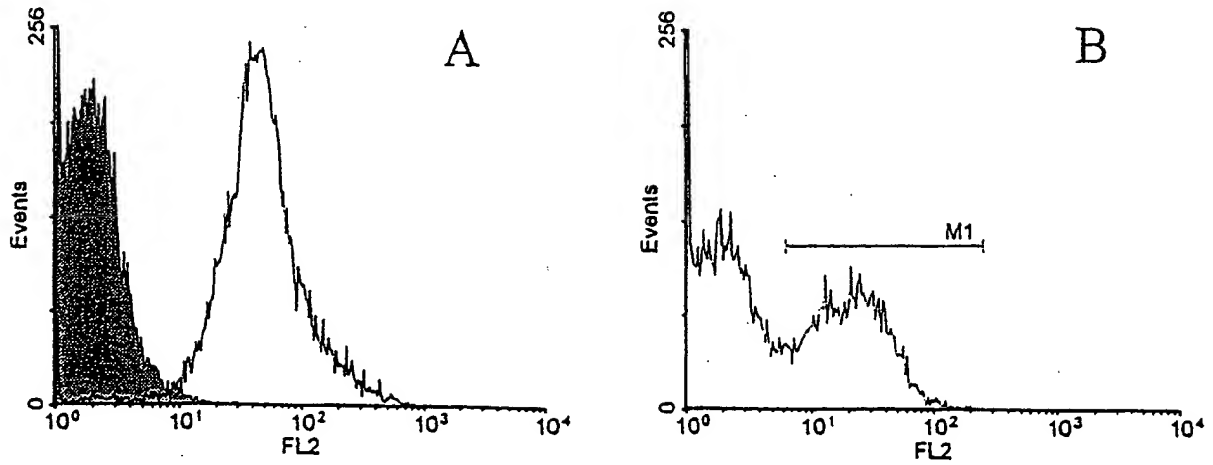

Fig 1. Collagen-mediated activation of human platelets. Panel A: calf skin collagen type I (10 μg/ml) was incubated with gel-filtered human platelets at 37°C. Alpha-granule secretion in response to collagen was monitored with an anti-P-selectin antibody (biotinylated G5); bound G5 was detected with phycoerythrin-streptavidin (PE-SA, FL_2, abscissa). The shaded area represents an FL_2 histogram for control platelets, and the solid line indicates the FL_2 histogram for collagen-activated platelets. Panel B: platelets were activated with an intermediate collagen concentration (1 μg/ml) and stained for P-selectin expression as detailed. Note that only 38% of the platelets (gate M1) responded to this submaximal collagen concentration.

activation events, a pre-incubation step would be expected to modify the platelet response. These data suggest that oligomerization during the time scale of these assays is not a significant variable. Secondly, possible collagen-induced aggregation of platelets was monitored by co-incubation with an equal number of washed erythrocytes. The ratio of platelets to erythrocytes before and after incubation with various collagen types was constant, indicating that no significant platelet aggregation occurred (data not shown).

RESULTS

Incubation of human platelets with either 10 μg/ml or 1 μg/ml of calf skin type I collagen resulted in expression of cell

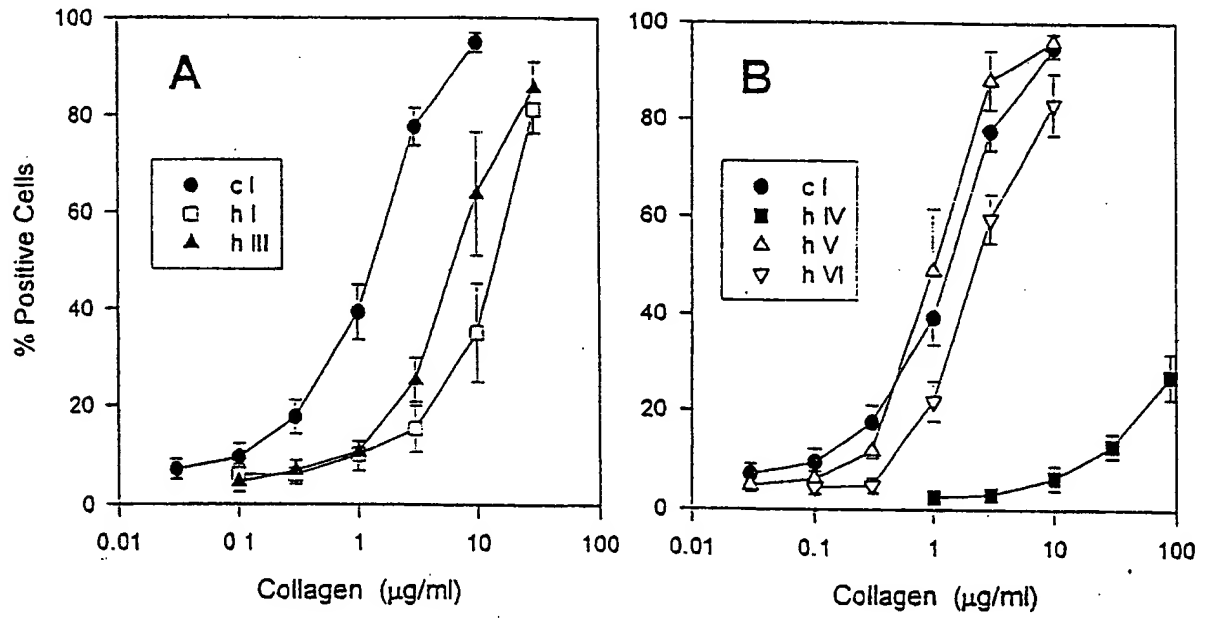

Fig 2. Dose–response curves for collagen stimulation of human platelets. Graded doses of various collagen types were reacted with gel-filtered, human platelets as detailed in Fig 1. The percentage of platelets positive for surface P-selectin was then quantitated (ordinate) and plotted versus collagen concentration (abscissa). Panel A represents calf skin collagen type I (solid circle), human type I (open square) and human type III (solid triangle); panel B represents calf skin collagen type I (solid circle), human type IV (solid square), human type V (up triangle) and human type VI (down triangle). Note that five of the six collagen types elicited significant α-granule secretion. Data represent mean ±1 SD; $n = 3–6$.

surface P-selectin on 97% or 38%, respectively, of all cells (Fig 1). The response of platelets to collagen was further analysed with graded doses of each collagen type over a concentration range of 0·03–90 μg/ml (Fig 2). Of the six different collagens tested, calf skin type I, human type V and human type VI were the most active. Human type III and human type I clearly elicited α-granule secretion although with apparent EC_{50} values approximately 10-fold higher than that observed with calf skin type I. And finally, human type IV collagen was not appreciably active in the concentration range utilized here.

Strong platelet agonists are also known to result in a redistribution of GPIIb/IIIa to the platelet surface (Nurden, 1997). The effect of graded doses of calf skin collagen type I on the surface level of GPIIb/IIIa in human platelets is demonstrated in Fig 3. In panel A the mean fluorescence for FITC-AP2, an anti-GP IIb/IIIa monoclonal, demonstrates a collagen-dose–response curve similar to that for α-granule secretion. In panel B this response was further dissected in a dual-labelling experiment by measuring the mean FITC-AP2 fluorescence for P-selectin-positive and P-selectin-negative platelets at each collagen concentration. These data demonstrated that the mean FITC-AP2 fluorescence for the P-selectin-negative population remained constant at the pre-treatment level whereas the P-selectin-positive population had an elevated level of fluorescence. This observation suggests that GPIIb/IIIa redistribution occurred concurrently with α-granule secretion. The ability of various collagen types to induce redistribution of GPIIb/IIIa is shown in Fig 4. Similar to the results with P-selectin expression, collagen types I, III, V and VI elicited GPIIb/IIIa redistribution whereas type IV did not.

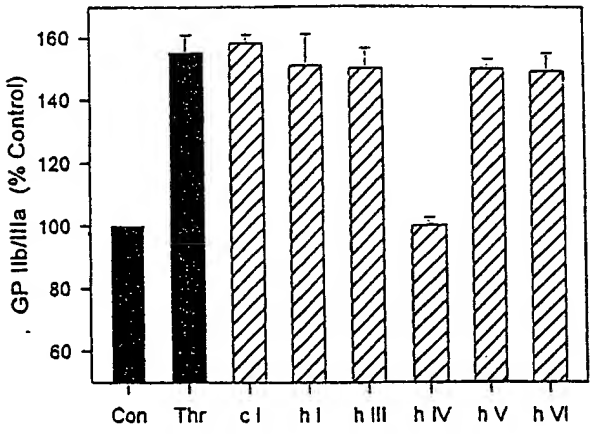

Fig 4. Effect of different collagens on GPIIb/IIIa redistribution. Gel-filtered human platelets were maximally stimulated with various collagen types and then stained with FITC-AP2. The mean fluorescence (ordinate) is shown for each collagen type (hatched bars); a quiescent control (Con) and thrombin-stimulated (Thr, 0·5 U/ml) sample are also shown for comparison (solid bars). Collagen types are identified by roman numerals; 'c' and 'h' signify calf and human, respectively. Data represent mean ±1 SD; $n = 3$.

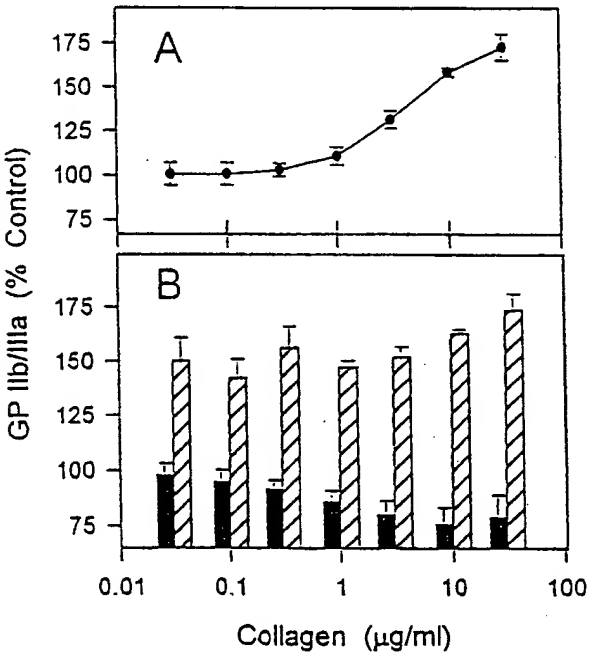

Fig 3. Effect of collagen activation on surface GPIIb/IIIa levels. Gel-filtered human platelets were reacted with graded doses of calf skin collagen type I and then labelled with FITC-AP2, an anti-GPIIb/IIIa monoclonal, and biotin-G5 to detect surface P-selectin. In panel A mean FL_1 fluorescence (FITC-AP2) for the total population is presented. Note the collagen-induced increase in surface exposed GPIIb/IIIa. In panel B the same samples depicted in the first panel were analysed for P-selectin expression, and the mean FL_1 for the P-selectin-positive (hatched bars) and P-selectin-negative platelets (solid bars) was determined. Note that the mean fluorescence for the GPIIb/IIIa antibody did not change from starting values for the P-selectin negative population whereas the P-selectin-positive population always had a high anti-GPIIb/IIIa fluorescence. Data represent mean ±1 SD; $n = 6$.

Activation of GPIIb/IIIa to its fibrinogen binding conformation is promoted by both strong (e.g. thrombin) and weak (e.g. ADP) agonists (Ware & Coller, 1995). Utilizing PAC-1, a monoclonal antibody which recognizes the activated conformation of GPIIb/IIIa (Shattil *et al*, 1987), we examined the ability of the various collagen types to activate human platelet GPIIb/IIIa. Fig 5 demonstrates that human type VI and calf skin type I were the most efficient in activating GPIIb/IIIa, whereas human collagen types I, III and V only activated GPIIb/IIIa at higher concentrations. Human type IV collagen resulted in essentially no change in PAC-1 binding. Even though there is no evidence that collagens directly bind to activated GPIIb/IIIa (De Groot & Sixma, 1997; Kehrel *et al*, 1998), there are reports that collagens can indirectly interact with this integrin (Coller *et al*, 1989). It was therefore necessary to consider the possibility that collagen might interfere with PAC-1 binding to activated GPIIb/IIIa. However, the fact that increased levels of these collagens potentiated PAC-1 binding (Fig 5) makes it unlikely that collagen was acting as a competitive inhibitor of PAC-1.

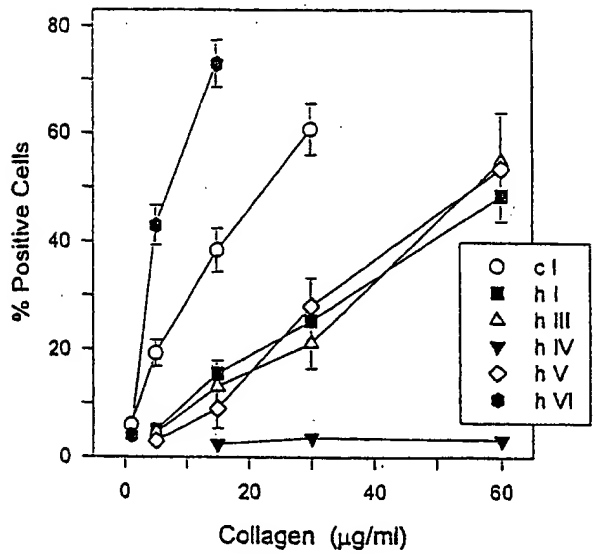

Fig 5. PAC-1 binding to platelets stimulated with collagen. Human platelets were activated with various collagen types and then analysed with PAC-1, an activation-dependent anti-GPIIb/IIIa monoclonal. The percentage of platelets positive for PAC-1 is indicated on the ordinate. The collagen types include: calf skin type I (open circle), human type I (solid square), human type III (open triangle), human type IV (solid triangle), human type V (open diamond) and human type VI (solid hexagon). Data represent mean ± 1 SD; $n = 3$.

DISCUSSION

In vivo platelet adhesion to collagen exposed in ruptured blood vessels occurs under flow conditions, often at high shear. As a result, most experimental analyses of the collagen–platelet interaction have mimicked this natural process, utilizing artificial flow systems with variable shear rates (Sixma *et al*, 1997). Although these experiments are informative for many aspects of the collagen–platelet interaction, there are some parameters which do not readily lend themselves to analysis under such conditions. For example, the ability of various collagen types to promote glycoprotein redistribution as well as integrin activation is difficult to assess.

In this report we describe a technique which allows analysis of secondary activation events resulting from a fluid-phase interaction between collagen and platelets. With this system, collagens type I, III, V and VI elicited P-selectin expression and up-regulation of surface GPIIb/IIIa, although with different efficiencies for the individual collagen types. Human collagen type IV, in the concentration range examined, did not generate a significant response. Additionally, calf skin type I and human type VI collagens were the most efficient at eliciting activation of GPIIb/IIIa to its ligand binding conformation (Fig 5); however, higher collagen concentrations were generally required for GPIIb/IIIa activation than for either α-granule secretion or up-regulation of surface GPIIb/IIIa (Fig 2). The differential response of human platelets to the various collagen types is summarized in Table I. Although it may be tempting to compare the activating potential of these various collagens and their respective platelet-adhesive capabilities (Saelman *et al*, 1994), any correlation appears tenuous.

The different reactivities of the various collagen types may depend on one or more factors. (1) The individual collagen types may have different affinities for the relevant receptor. (2) Different receptors or sets of receptors may be responsible for responses with the various collagen types. (3) The collagens themselves may be structurally different with regards to the degree of oligomerization under the assay conditions utilized. Although it is clear that none of the collagens examined were sufficiently polymerized to interfere with flow cytometric analysis, we have not at this time conclusively addressed the question of oligomeric structure for each collagen type. However, ultracentrifugation of the collagen stock solutions did not affect their stimulatory properties, indicating that macropolymers did not form in the collagen stock solutions. In addition, a 10 min pre-incubation of neutralized collagen at the low protein concentrations utilized here did not modify the degree of platelet activation (see Methods). This finding is in agreement with a previous study examining the kinetics of collagen fibril formation which demonstrated a considerable lag time for polymerization at neutral pH (Williams *et al*, 1978).

The dose–response curves demonstrated in Fig 2 are strikingly similar to those observed with other agonists such as thrombin (Peng *et al*, 1994). The basis for differential reactivity within a platelet population is unclear (Thompson & Jabubowski, 1988). For thrombin, some of the variability

Table I. Summary of activation characteristics of several collagen types with human platelets.

	Agonist						
		Collagen type					
Activation parameter	Thrombin	Calf skin I	Human I	Human III	Human IV	Human V	Human VI
P-selectin expression	++	++	+	+	±	++	++
GPIIb/IIIa up-regulation	++	++	+	+	±	++	++
GPIIb/IIIa activation	++	++	+	+	−	+	++

The response of platelets to the various collagen types was monitored as detailed in Methods.

can be attributed to age differences (Peng *et al*, 1994); there are, however, other factors controlling reactivity which remain unknown regardless of the agonist.

Finally, the variation in concentration dependence for expression of different activation markers with a specific collagen type was unexpected. For example, type V collagen, which was among the strongest activators for eliciting P-selectin expression, was only a modest activator of GPIIb/IIIa. On the other hand, type VI collagen was able to elicit both changes at almost the same effector concentration. These observations indicated that α-granule secretion and GPIIb/IIIa redistribution were separate events from GPIIb/IIIa activation.

Activation events reported here represent the final steps of intracellular signalling pathways initiated by various collagens. Studies with other integrins have shown that receptor occupation can result in intracellular phosphorylation reactions culminating in platelet activation (Shattil *et al*, 1994), and occupation of the collagen receptor(s) is known to elicit a number of intracellular events, including phosphorylation of syk, src and PLCγ2 (Keely & Parise, 1996). The role of these events in expression of the secondary activation markers monitored here is not clear; however, it does appear likely that more than one receptor, and therefore multiple intracellular signalling pathways, are involved.

The current experiments have utilized a technique which allows a fluid phase interaction between human platelets and various collagen types. The strength of this procedure is the ability to utilize flow cytometry for analysis of secondary activation events resulting from collagen stimulation. Although this methodology has shed new light on the reactivity of the various collagen types, complementary experiments investigating the receptors and intracellular signalling utilized by the different collagens are required to fully elucidate the molecular basis for these differential reactivities.

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Type IV and type "A-B" collagens do not elicit platelet aggregation or the serotonin release reaction

ROBERT L. TRELSTAD and ANGELINA C A CARVALHO* *Boston, Mass.*

Human collagens were isolated from kidney, lung, skin, aorta, cartilage, and placenta. Five different types were obtained, including two new molecular species, one characteristic of basement membranes, or type IV collagen, and the other the recently described "A-B" collagen derived from fetal membranes. All the collagens were purified and separated by combination of heat-gelation fractionation and salt fractionation. In neutral solution at 37° neither type IV nor type "A-B" collagen elicited platelet aggregation or ^{14}C-serotonin release. Preincubation of platelets with both types IV and "A-B" collagen did not inhibit aggregation upon subsequent addition of collagen types I, II, or III. (J LAB CLIN MED 93:499, 1979.)

Abbreviations: sodium dodecyl sulfate (SDS), segment-long-spacing crystallites (SLS crystallites), carboxy methyl cellulose (CM cellulose)

The aggregation of platelets in vitro by the interstitial collagen types I, II, and III has been demonstrated,[1, 2] and although the mechanism of this reaction is not fully understood, it is principally associated with the capacity of these collagens to undergo polymerization to higher-order multimeric structures.[3–6] Recently additional vertebrate collagen species have been identified, one of which is known to be of basement membrane origin, designated type IV collagen,[7, 8, 12] and the other of which has been temporarily designated type "A-B".[9, 10] Type "A-B" consists of A and B chains which may be present in two separate forms (A_3 and B_3) or together (AB_2). This has not been firmly established, nor has its or their possible derivation from basement membranes.

Collagen types IV and "A-B" can be separated from the other collagen types on the basis of their unusually high solubility at both acid and neutral pH[9, 10] and by their inability to spontaneously aggregate at neutral pH when heated to 37° C.[8] We report here that collagen types IV and "A-B" do not elicit platelet aggregation and the serotonin release reaction nor do they inhibit such platelet reactions induced by the other interstitial collagens.

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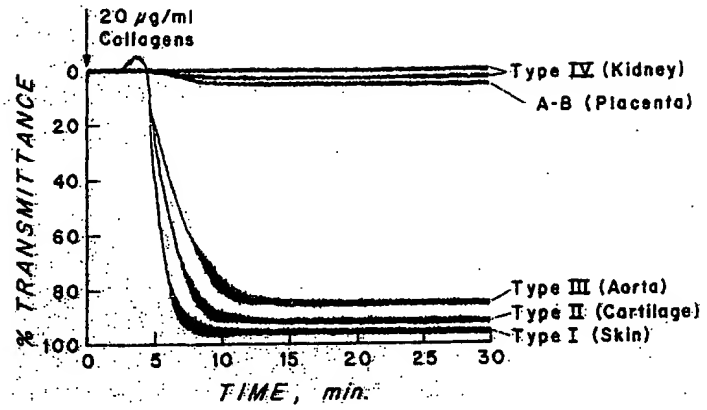

Fig. 1. Aggregometry of platelets upon exposure to the five different human collagens as indicated Prolonged incubation with types IV and "A-B" (or at concentrations of 100 μg/ml) elicited less than a 10% response, whereas the collagen types I, II, and III all reacted readily at concentrations as low as 2 μg/ml.

Materials and methods

Samples of aorta, lung, kidney, skin, and cartilage were obtained at autopsy within 12 hr of death and placental membranes at time of delivery. Following homogenization in 0 5M acetic acid at 4° C the specimens were subjected to limited proteolytic digestion with pepsin (2× crystalized, 20 mg/gm wet weight; Worthington Biochemical Corp., Freehold, N. J.) for 72 hr at 4° C. The solubilized collagens were centrifuged, and the extract was neutralized with NaOH to pH 7 5 to inactivate peptic activity The entire collagen mixture was then reprecipitated by rapid addition of solid NaCl to a final concentration of 20% w/v, and the precipitate was collected by centrifugation. The precipitate was then redissolved in 0 16M potassium phosphate buffer, pH 7.6, and dialyzed against the same to remove the NaCl. The solution was then heat-gelled at 35° C for 16 hr, after which it was centrifuged and separated into gelled and nongelled fractions. The types IV and "A-B" collagens do not heat-gel under these conditions and were subsequently precipitated by the addition of 20% NaCl w/v to the heat-gel supernatant.[8] The collagens were resolubilized in the phosphate buffer, dialyzed against buffered 2.5M NaCl for 24 hr at 4° C, and centrifuged; the supernatant fraction containing either the type IV or "A-B" collagen was dialyzed against 0.1M acetic acid and stored in solution. Type IV collagen was the major species obtained by this fractionation scheme from the kidney, whereas type "A-B" was the major species obtained from the placental membranes. Collagen types I, II, and III, present in the heat-gel precipitate of skin, cartilage, and aorta, respectively, were resolubilized in phosphate buffer and fractionated by salt precipitation as previously described.[11] The fractionated collagens were dialyzed against 0.1M acetic acid and stored in solution. All collagens were prepared for aggregometry study by dialysis against 0.2M NaCl, 0.05M Tris-HCl, pH 7 5, and solution concentrations were determined by 6N HCl hydrolysis of aliquots and measurement of the trans-4-hydroxyproline content on an amino acid analyzer

Human placental membranes were homogenized and pepsinized as above and then divided into two aliquots, one of which was subjected to the heat-gelation fractionation procedure and the second dialyzed against 2.5M sodium chloride, 0.08M phosphate buffer, pH 7.6, overnight. The heat-gel–fractionated material was handled as described above, and the salt-fractionated material following dialysis was centrifuged and separated into a 2.5M precipitable and 2.5M NaCl-soluble fractions. The "A-B" collagens present in the heat-gel supernatant and the 2.5M NaCl-soluble fraction were reprecipitated by rapid addition of 20% NaCl w/v, and the precipitates were resolubilized in 0 1M acetic acid and dialyzed versus the same and stored.

Purity of the various collagen types was determined by amino acid analysis, electrophoresis on polyacrylamide gels using both an acid-urea system and SDS, chromatographic properties on CM-cellulose, and electron microscopic patterns of SLS crystallites as described in detail elsewhere.[8–10, 12]

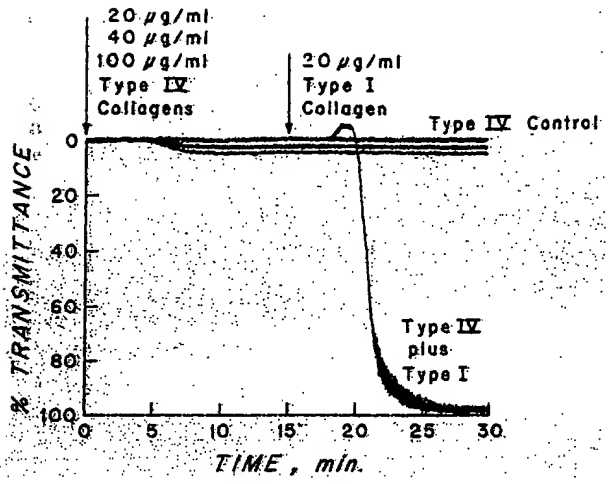

Fig. 2. Aggregometry of platelets after 15 min preincubation with type IV collagen and subsequent addition of type I. The reaction in respect to extent and lag time for type I was unchanged Similar negative results were obtained by preincubation with type "A-B" collagen.

Table I. ^{14}C-serotonin release elicited by exposure of prelabeled platelets to collagens at three different concentrations*

—Collagens—	20 μg/ml (% of uptake)	40 μg/ml (% of uptake)	100 μg/ml (% of uptake)
Type I (skin)	98	—	—
Type II (cartilage)	90	—	—
Type III (aorta)	88	—	—
Type IV (kidney)	0	5	10
A-B (placenta)	4	7	12

Mean of 8 determinations
*Collagen types I, II, and III elicit release quantitatively, whereas types IV and "A-B" do not, even at high collagen concentrations.

The melting point of the collagens was determined by measurement of changes in the circular dichroism of neutral collagen solutions, pH 7.6, at 221 nM in a Cary-60 spectropolarimeter by incrementally increasing the temperature of the solution in a 1 mm path-length jacketed cell.[12]

The collagens were adjusted to a final concentration of 0.8 to 1 0 mg/ml, and from 10 to 100 μl of these solutions were added to 0 5 ml of platelet-rich plasma for aggregation studies

Venous blood was collected from normal volunteers in plastic syringes, and 9 volumes of sample were added to 1 volume of 3.8% sodium citrate, mixed, and centrifuged at room temperature for 10 min at 100 × g [13] The platelet-rich plasma, containing 200,000 to 300,000 platelets/μl, was assayed for aggregation with the method described by Born.[14] In some experiments normal platelet-rich plasma was preincubated with ^{14}C-serotonin (0 8 μCi/ml; New England Nuclear, Boston, Mass.) for 25 min at 25° C The release of the radioactive material from the platelets was induced by exposure to the various collagens. The reaction was monitored by continuous recording in an aggregometer (Bio/Data Corp, Willow Grove, Pa.) for 20 min at 37° C After centrifugation of the reactants for 15 min at 4° C, 0 1 ml of the supernatant fraction was added to 10 ml of liquid scintillation (Scintisol Complete; Isolab, Inc., Akron, Ohio), and the radioactivity was counted (Beckman scintillation counter; Beckman Instruments, Inc., Palo Alto, Calif.).[15]

Blood collection and tissue acquisition were done according to protocols approved by the Human Studies Committee of the Massachusetts General Hospital.

Results

Complete aggregation of the platelets and near-total release of prelabeled ^{14}C-serotonin were achieved by collagen types I, II, and III prepared respectively from skin, costal cartilage, and aorta (Fig. 1; Table I). Complete reaction was achieved with collagen types I, II, and III with as little as 2 μg/ml collagen, but most experiments were done with 20 μg/ml to achieve the control curves. Attention was not directed at the relative reactivities of collagen types I, II, and III or to the effects of preheating the solutions to promote aggregation. Few differences were therefore noted in the reactivity of these interstitial collagen types

Collagen type IV derived from kidney, lung, aorta, and skin and the type "A-B" collagen derived from placenta did not elicit platelet aggregation or the release reaction in the concentration range of 20 to 100 μg/ml collagen (Fig 1; Table I). The incubation with platelets was allowed to continue for as long as 30 min in some circumstances, and at most 10% aggregation and 12% release were observed.

When platelets were preincubated with either type IV or type "A-B" collagen for up to 30 min at concentrations from 20 to 100 μg/ml, there was no subsequent inhibition of platelet aggregation, in that addition of collagen types I, II, or III at concentrations from 2 to 20 μg/ml elicited prompt aggregation without substantial changes in lag time or extent of aggregation (Fig. 2).

There were no apparent differences between type "A-B" collagens prepared by heat-gelation fractionation and those obtained by salt fractionation. Both components failed to elicit aggregation following a 20 min incubation and stirring (1200 rpm), and neither preparation showed inhibition of subsequent platelet aggregation following addition of types I, II, or III.

Denaturation of collagen types I, II, and III by gentle heat treatment (10 min at 50° C) completely eliminated the capacity of these collagens to elicit aggregation and release reaction when used at the concentration of 20 to 40 μg/ml. Heat denaturation of the type IV collagen and "A-B" chains showed no change in their nonreactivity.

The purity of the types IV and "A-B" collagens was determined by the amino acid composition of the native material, SDS-gel electrophoresis, and SLS crystallite formation.[8–10, 12] In all preparations contamination of the types IV and "A-B" chains with types I, II and III was not detectable on the SDS gels.

The native state of the types IV and "A-B" collagens was indicated by their resistance to peptic digestion under native conditions; their susceptibility to peptic digestion following brief heat denaturation; their capacity to precipitate at both low and high ionic strengths (0.01M Na_2HPO_4 and high salt, 20% NaCl); the capacity of type "A-B" collagen to form SLS crystallites; and the pronounced change in circular dichroism at 221 nM in both collagen types between 38° and 41° C.

Discussion

These observations indicate that collagens derived from basement membranes (type IV) and placental membranes (type "A-B") do not elicit platelet aggregation or the release reaction nor do they inhibit those reactions by additionally added interstitial collagens. Previous studies have indicated that the aggregation and release reaction with collagen types I, II, and III depend largely on the acquisition of higher-order states of polymerization beyond that present in the collagen monomer.[1–6] Quaternary structure, perhaps of a specific order, is therefore important for part of the platelet-collagen interac-

tion, although it is apparent that interactions with denatured chains or collagen polypeptides from some animal species can occur.[16] The role of carbohydrate side chains in the platelet-collagen reaction, on reassessment,[6, 17] appears less important than originally thought, and our observations are also inconsistent with this model because type IV collagen contains approximately 10% to 12% carbohydrate by weight.[8]

The types IV and "A-B" collagens do not heat-gel into opaque polymeric gels when heated under physiological conditions at 37° C The circular dichroism studies conducted on these two collagens indicate that the native triple helix is not destroyed at the temperatures employed in the heat-gelation isolation procedure, and ultrastructural studies indicate that heat-gelled solutions of types IV and "A-B" do contain aggregated structures but not striated fibrils. The nongelling behavior of these new collagen species and their nonreactivity with platelets are additional evidence, albeit indirect or negative, for an important role of an ordered fibril structure in the elicitation of the platelet response It should be emphasized that the nongelled solutions of both types "A-B" and IV do contain aggregated structures as do solutions of Type I collagen in early stages of fibril formation.[4, 18] Differences between the gelling and nongelling collagen types are therefore apparently at the level of aggregate interactions leading to ordered fibrillar structures. Characterization of some of the structural features of these intermediate aggregate forms will be necessary in subsequent studies of the platelet-collagen interaction, and physical techniques such as laser light scattering will likely provide more detailed information about the early stages of collagen aggregation and differences among the various collagen types [19]

The inability of either type IV or "A-B" collagen to inhibit platelet aggregation or release is consistent with the recent report of Santoro and Cunningham[6] that monomeric collagen in excess does not inhibit aggregation by polymeric forms. In contrast, the inhibition of platelet aggregation which occurs with C1q[20] and which is dependent on the collagen-like portion[21] is not seen with types IV and "A-B," in that rapid aggregation occurs upon addition of lesser (2 μg/ml) or equivalent amounts (20 μg/ml) of the interstitial collagens as used in the C1q studies.[21]

A number of previous reports have indicated a low level or nonreactivity of platelets with sonicated basement membranes isolated from heart valves and glomeruli.[22, 23] Adherence of the platelets to sonicated glomerular basement membranes, however, does occur without causing aggregation or the release reaction.[23] Our preparations of types IV and "A-B" collagens do not contain the noncollagenous constituents which are present in such sonicated glomeruli. The fact that these purified collagens do not elicit the platelet aggregation and release reaction suggests that the earlier reports with sonicated basement membranes indicating nonaggregation do not reflect masking of reactive sites on the type IV (or "A-B") collagen by other basement membrane constituents. It remains to be determined whether isolated type IV or "A-B" collagen can bind platelets when assayed by affinity column techniques.

The identification of a collagen species as being derived from a basement membrane depends on the comparison of its composition with those isolated from the renal glomerulus and lens capsule. The compositions of the collagens we have used, derived from the kidney, lung, and aorta, closely resemble that obtained from purified isolated glomeruli, and antibodies against the kidney-derived material react with lung, vascular, and epidermal basement membranes by indirect immunofluorescence. The type "A-B" collagen has been tentatively identified as a basement membrane collagen because of its relatively high content of 3-hydroxyproline and its elevated levels of glycosylated hydroxyl-

ysine.[9] This identification, however, remains tentative until further specific localization studies using type-specific antibodies have been completed.

The accumulated data concerning nonreactivity of platelets with basement membranes or their collagenous components suggest that the immediate subendothelial matrix which is initially exposed upon loss of endothelial integrity is a relatively nonthrombogenic surface. The microfibrillar and collagen fibrillar structures which lie beneath the basement membrane, however, are quite potent aggregators of platelets, and it is presumably these structures which, when exposed, elicit platelet aggregation.[24]

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Platelet-Reactivity of Isolated Constituents of the Blood Vessel Wall

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Key Words. Arterial collagens · Collagen polymorphism · Platelet aggregation · Platelet adhesion · Elastin · Microfibrillar protein

Abstract. Collagens I and III, in fibrillar form, bound platelets equally well; both readily induced platelet aggregation. In contrast, collagens IV and V, although pretreated as collagens I and III to induce fibrillogenesis, failed to produce aggregation. No binding of platelets was detected. Lens capsule, containing collagen type IV *in situ*, was also inactive. Inactivity appears due to the lack of an appropriate quaternary structure since segment-long-spacing (SLS) aggregates of collagens IV and V, as of type I, induced aggregation.

Elastin and its associated microfibrillar element did not aggregate platelets; some binding of platelets to elastin only was observed.

The interaction between blood platelets and elements of the connective tissue resulting in platelet aggregation is regarded as a basic feature of the haemostatic mechanism. More particularly, the potent platelet-aggregating activity of connective tissue collagen fibres (located either directly in the damaged blood vessel wall or elsewhere at the site of injury) is considered of fundamental importance in this mechanism. The interaction of platelets with the subendothelial connective tissue constituents of the vascular wall, particularly collagen, is also considered to play a central role in thrombogenesis as well as perhaps in atherogenesis – for review, see (17) and references therein.

With the recognition in recent years that collagen occurs not as a single molecular entity but as a number of genetically distinct, structurally different

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subtypes, there was an evident need to re-examine the activity of collagen fibres towards platelets in the context of collagen polymorphism. This has been undertaken by us and others and the following results are reviewed.

Chung and Miller (11) and *Trelstad* (36) first established that the blood vessel wall contains more than one collagen when they demonstrated independently the presence of substantial amounts of collagen type III as well as type I. The former collagen, of chain composition $[\alpha 1(III)]_3$, appears to be relatively abundant in the more extensible tissues such as vascular wall, skin and uterus. Type I, of chain composition $[\alpha 1(I)]_2\alpha 2$, is the most commonly occurring type of collagen and is essentially the sole species in bone and tendon. The presence of these two collagens in arterial tissue has been confirmed in a number of laboratories and their synthesis by cultured medial smooth muscle cells has been demonstrated (4, 9, 24, 25, 27, 30, 33). Immunofluorescent studies (16) have indicated that in the young blood vessel wall, type III collagen without type I occurs in the immediate subendothelial (intimal) space and that both occur in the media, type III in association with the elastic laminae and type I in the space between the laminae and with an increasing preponderance of type I in the adventitial direction. In contrast, in the older vessel, the diffusely thickened intima contains much more type I than type III (*Barnes,* unpubl.) and likewise the intimal plaque contains largely type I with relatively little type III (28).

The studies of *Trelstad* (36) have indicated that type IV collagen identified originally in classical basement membrane structures such as kidney glomerular basement membrane and lens capsule is also present in the blood vessel wall. Its location is presumed to be in vascular basement membranes, particularly that underlying the endothelium. The precise structure of type IV collagen is as yet undetermined. Its origin in the vascular wall from endothelium, which is considered to formulate the adjacent basement membrane, seems likely and indeed cultured endothelial cells have been shown to synthesize collagen (18, 22, 26) that has been described as of type IV in nature (18, 22). However, endothelial cells have also been shown to synthesize interstitial collagens in culture (6) and the possibility exists, therefore, that these cells may also be the site of origin of interstitial collagens in the intima such as the types I and III already alluded to. In general, however, the latter collagens are considered to arise from smooth muscle cells that have migrated from the media into the intimal space.

More recently another collagen believed also to be associated with basement membranes has been identified, initially in fetal membranes (10, 12, 13). This collagen, type V, containing α-chains which have been designated as A and B chains and which occur within the molecule probably in a ratio of 1:2, appears

also to occur in vascular tissue. *Chung et al.* (12) have isolated B chains (but without A chains) from the media. *Mayne et al.* (27) have detected the synthesis of both A and B chains by medial smooth muscle cells, together with a collagenous peptide of 45,000 daltons, the origin of which is unclear. *Chung et al.* (12) have also detected in the intima a high molecular weight collagenous constituent, that upon reduction yields a peptide of 55,000 daltons. The relationship of this particular constituent to the other collagen types so far described is as yet uncertain, although it is speculated that it is a component of the endothelial basement membrane.

There is, thus, a variety of collagenous elements in the blood-vessel wall distributed in a highly specific manner. As a consequence, the type of collagen exposed to blood platelets following injury is likely to depend upon the precise nature of that injury and the character of the platelet response to injury will depend upon the reactivity of the particular collagen(s) exposed.

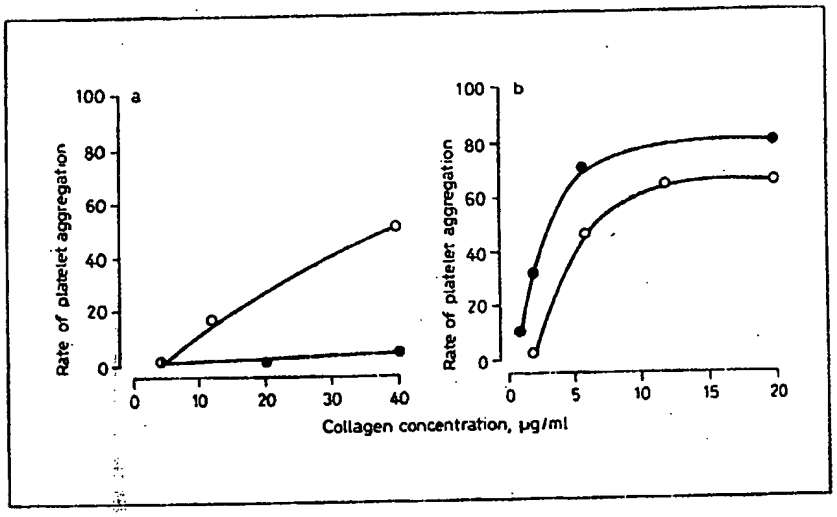

Fig. 1. Platelet aggregation by collagens types I and III from chick skin. Platelet aggregation was measured in 0.1 ml samples of human citrated platelet-rich plasma by observing changes in optical density following the addition of known amounts of collagen. The rate of aggregation was obtained from the maximum rate of change in light transmission. (a) Platelet responses following addition of collagens in solution (10 µl or less) in 0.1 *M* acetic acid. ○ = type III; ● = type I. (b) Responses following addition of collagens in fibrillar suspension: collagen solutions were preincubated with an equal volume of cell-free plasma. From *Barnes et al.* (3) by permission of the Biochem. J.

The activity of some of these different types of collagen towards platelets will be described in the forthcoming sections of this article, not only as regards their possible aggregatory activity, the importance of which has already been stressed, but also from the point of view of their ability simply to permit platelet adhesion irrespective of any ability to induce aggregation. A number of studies have indicated that platelets can adhere to the vascular basement membrane (see 7). In particular, the electron microscopic studies of *Baumgartner et al.* (7) have demonstrated that when the endothelium is removed experimentally, platelets attach to the denuded area. There is initially a rapid build-up of these cells but this soon disperses leaving a simple monolayer of platelets bound to the subendothelium. This is thought to be a process that may occur as a normal physiological event; the implication is that a constituent of the subendothelial tissue permits platelet adhesion without aggregation. Such a constituent could be located conceivably in the basement membrane (e.g. collagens types IV or V) or alternatively in elastic fibres located in the immediate subendothelial space in areas where the basement membrane is interrupted (7, 35). We have been concerned, therefore, not only with the platelet reactivity of arterial collagens but also with the elements of the elastica, namely the protein elastin and its associated microfibrillar glycoprotein identified by electron microscopy by *Ross and Bornstein* (31).

Platelet Reactivity of Different Collagens

Interstitial Collagens

Several groups have now examined the relative platelet reactivity of collagens types I and III (1, 3, 21, 32). When these two collagens are presented to platelets in solution, i.e. in monomeric form, activity (aggregation) is observed only after a delay consistent with the occurrence of fibrillogenesis prior to aggregation. Type III collagen appears considerably more active than type I. However, if the collagens are presented in polymeric form as preformed fibrils (by preincubation of solutions at 37 °C, in platelet-poor plasma for example), the lag period is much reduced, platelet aggregatory activity of both types is greatly enhanced and the two collagens exhibit a comparable order of activity (fig. 1, 2). These observations are entirely in accord with the concept that a highly ordered collagen quaternary structure is an essential prerequisite for the induction of platelet aggregation by collagen (23). The greater activity of type III relative to type I solutions presumably reflects a greater facility on the

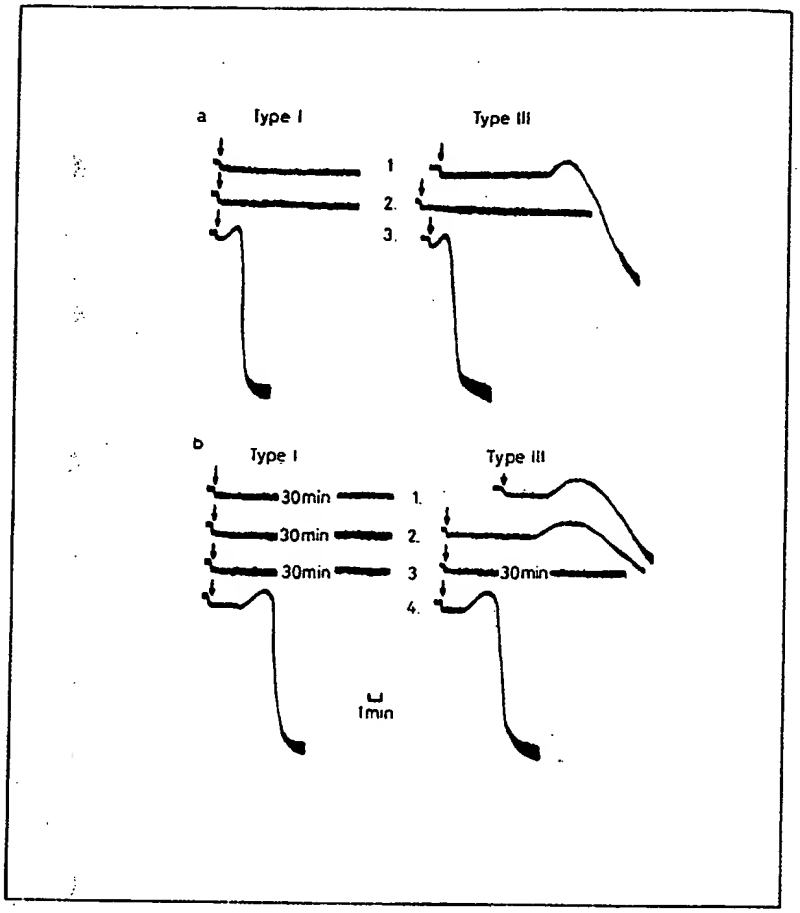

Fig. 2. Platelet aggregation by collagens types I and III from human aorta and chick skin. Platelet aggregation was measured as described in figure 1. The traces shown represent actual changes in optical transmission following addition of collagen (at the arrow) to a final concentration of 20 μg/ml. (a) Chick collagen: (1) Addition of collagen in solution in 0.1 *M* acetic acid. (2) Addition after preincubation in an equal volume of 0.9% NaCl; treatment under these conditions yields inactive precipitates. (3) After preincubation in 0.38 *M* Na_2HPO_4 or cell-free plasma; preincubation under these conditions yields highly potent native-type fibrils. (b) Human collagen: (1) As a 1. (2) Addition of collagen after preincubation in an equal volume of cell-free plasma; the results suggest an inhibitor of human collagen fibrillogenesis in human plasma. (3) After preincubation in an equal volume of 0.9% NaCl. (4) After preincubation in an equal volume of 0.38 *M* Na_2HPO_4. From *Barnes et al.* (3) by permission of Biochem. J.

Table I. Adhesion of platelets to various constituents of the extracellular matrix

Preparation		Percentage adhesion
Collagen (pig aorta)	type I polymer (i.e. native-type fibrils)	24.5 ± 2.25
	type I monomer	0
	type III polymer	25.5 ± 2.3
	type III monomer	0
Collagen (human placenta)	type IV	0
	type V	0
Collagen (bovine tendon)	type I polymer (from Ethicon Inc.)	77.8 ± 6.8
Elastin (pig aorta)		7.2 ± 1.9
Microfibrils (pig aorta)		1 ± 1.0

Adhesion was measured by the method of *Brass et al,* (8) as indicated in the text using a column of Sepharose 2B to which the constituent in question was covalently attached. The percentage adhesion refers to the proportion of the total number of platelets applied to the column that was retained by the column. Results are mean values ± SD of 4 to 6 determinations, each performed in triplicate.

part of the former collagen to undergo fibrillogenesis when incubated in monomeric form in plasma. The alternative explanation advanced by *Hugues et al.* (21) that type III collagen may, in fact, be the active species, and that the activity of type I preparations represents the presence of small amounts of type III as impurity in these preparations, seems to us improbable in view of the similar order of activity of the two collagens when in fibrillar form. Furthermore, the ability of fibrils of α(I) trimer, formed after reassociation of α1(I)-chains isolated chromatographically and free of type III chains, to aggregate platelets would confirm the intrinsic activity of type I collagen (2). *In vivo,* where types I and III are both likely to occur in the blood vessel wall as fibrils with the characteristic 67 nm periodicity, as observed by electron microscopy and indicative of the highly specific quaternary structure of interstitial collagens, we assume the two collagens will reveal a similar order of platelet reactivity.

We have undertaken binding studies using the technique described by *Brass et al.* (8) in which platelets, in the presence of EDTA to inhibit aggregation, are passed down a column of Sepharose 2B to which the collagen under investigation has been covalently attached. Our results (table I) indicate that both

collagens I and III in fibrillar form have a similar platelet-binding capacity, in agreement with the findings of *Fauvel et al.* (14) using a somewhat different assay procedure. Little, if any, activity was observed with either collagen in monomeric form. The greatest binding (and aggregating) activity we have observed has been with a highly polymerized, but nevertheless highly-dispersed, preparation of bovine tendon type I collagen (kindly supplied as a gift by Ethicon, Inc., Somerville, N.J.). This confirms our belief that the quaternary structure is the over-riding feature of the collagen molecule that governs both the extent of adhesion to platelets and the ability to induce their aggregation.

In view of the location of type III collagen without type I in the immediate subendothelial space on the inner surface of the internal elastic lamina (at least in the blood vessel wall where little, if any, intimal thickening has occurred), it has been suggested that this collagen species may be particularly important in thrombogenesis. Its detection in fresh thrombi is particularly interesting in this context (16).

Basement-Membrane Collagens

As already indicated, the subendothelial basement membrane is likely to contain, or be associated with, collagens types IV and V. In collaboration with Dr. *A.J. Bailey*, Meat Research Institute, Bristol, UK, and Dr. *J.L. Gordon*, Institute of Animal Physiology, Cambridge, UK, we have undertaken an examination of the platelet-reactivity of collagens IV and V isolated not directly from vascular tissue but more conveniently from other sources. Type IV collagen isolated from bovine anterior lens capsule and type V collagen isolated from either human placenta or bovine lung tissue failed to induce platelet aggregation even when solutions were preincubated in platelet-poor plasma at 37 °C or were dialyzed against 0.02 *M* Na_2HPO_4, procedures known to yield active fibrils (with a 67 nm periodicity) in the case of interstitial collagens (5). This suggested that these collagens either failed to precipitate (i.e. form fibrils) under the conditions employed or they formed amorphous fibrils lacking the required quaternary structure suitable for platelet reactivity. *Trelstad and Carvalho* (37) have also reported, briefly, on the inability of isolated basement-membrane collagens to induce platelet aggregation. We observed, likewise, that dispersions of intact lens capsule were inactive although they did in some measure inhibit platelet aggregation induced by interstitial collagen – figure 3 (5). We assume this lack of activity reflects the amorphous nature of basement membranes and the absence of collagen fibrils with a highly-ordered quaternary structure such as that characteristic of the interstitial collagens.

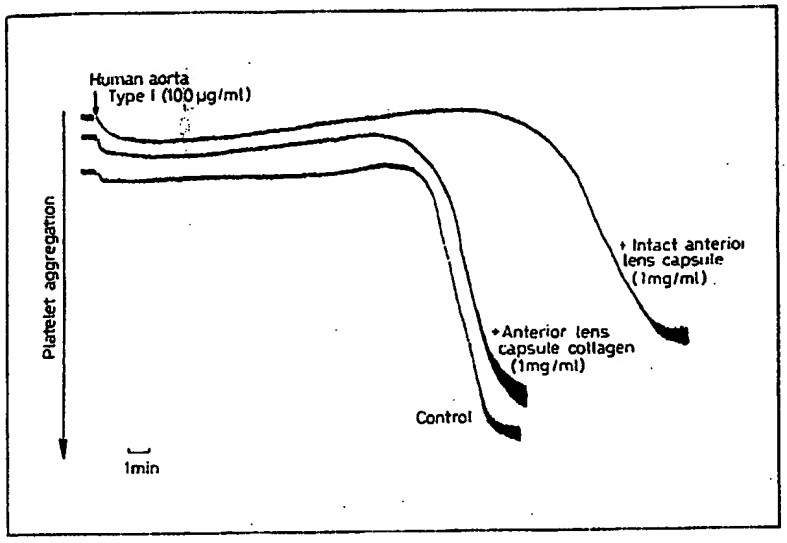

Fig. 3. Inhibition of collagen-induced platelet aggregation by intact bovine lens capsule. Platelet aggregation was measured as indicated in figures 1 and 2. A dispersion of lens capsules or a solution of purified type IV collagen from capsules was added (at the specified concentration) to platelet-rich plasma just prior to the addition of a solution of type I collagen from human aorta. Inhibition by the lens capsule preparation is indicated by both an increase in the lag period before aggregation commences and by a reduced rate of aggregation. From *Barnes and MacIntyre* (5) by permission of S. Karger AG, Basel.

In order to assess if the lack of activity of basement-membrane collagens was in fact due to the absence of a suitable quaternary structure, rather than to an intrinsic inability of these collagen types as such to induce aggregation, we examined the activity of SLS aggregates of collagens types IV and V and of type I. SLS aggregates were prepared as usual by dialysis against adenosine triphosphate (ATP). It was necessary to remove ATP prior to testing for platelet-aggregatory activity. SLS aggregates were, therefore, first stabilized by treatment with formaldehyde (38) and ATP then removed by dialysis. Collagen SLS aggregates of all types examined were found to be able to induce platelet aggregation, types IV and I being active around 10 µg/ml, and type V at about 35 µg/ml (fig. 4). There was, however, some variation in activity from preparation to preparation and we consider this may be due to variation in the amount of end-to-end polymerization of SLS aggregates during treatment with formaldehyde. It seems likely that stabilization with formaldehyde causes some associ-

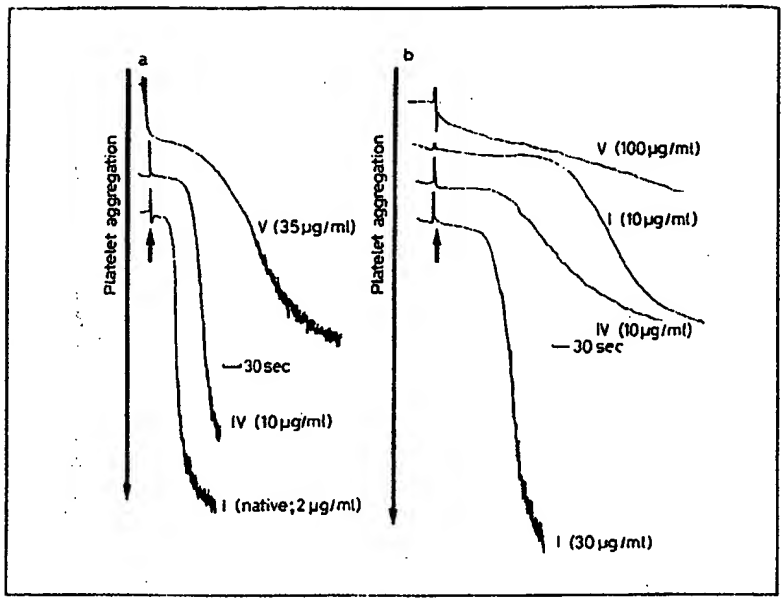

Fig. 4. Platelet aggregation by SLS aggregates of collagens types I, IV and V. Platelet aggregation was measured as in figures 1 and 2. The concentration of collagen added (at the arrow) is indicated. The results are for two separate experiments: (a) type I collagen (native-type fibrils) was a dispersion of highly-polymerized bovine tendon collagen from Ethicon Inc.; types IV and V collagens were isolated from human placenta and SLS aggregates prepared as described in the text. Concentrations are approximately the minimal for activity. (b) All as SLS aggregates: type I collagen was from rat tail tendon, types IV and V from human placenta.

ation of the aggregates (to form F-SLS) and it is these polymerized forms that are the active species. This would be in accord with the observation of *Wang et al.* (39) that simple SLS aggregates are not active and that a polymerized structure the equivalent of at least three molecules in length is necessary for the induction of platelet aggregation. Nevertheless, irrespective of the precise nature of the active polymer (SLS or F-SLS) our results clearly imply that it is the presence of an ordered quaternary structure rather than a type as such that determines the ability of collagen to aggregate platelets.

As mentioned earlier, basement membrane collagen (type IV), *in vivo*, is thought to possess an amorphous (or at least relatively disordered) structure which fails to exhibit, under the electron microscope, a periodicity comparable

to the 67-nm periodicity characteristic of the interstitial collagen fibre which reflects the highly-organized quaternary structure of the latter. The same may also be the case for type V collagen. If this is so, platelet aggregation by these collagens *in vivo* seems improbable. This would be in accord with the observations of *Huang and Benditt* (19) and *Huang et al.* (20), who found that preparations of human glomerular basement membrane, or the collagenous matrix derived from the membrane by pepsin digestion, do not cause platelet aggregation. Adhesion of platelets to the membrane appears attributable to a non-collagenous constituent. The inhibition of collagen-induced platelet aggregation by intact lens capsule we have observed could reflect interaction between platelets and a similar non-collagenous constituent.

Compatible with the notion that the collagen(s) of basement membranes is not involved in the reaction with platelets, we have detected no binding of platelets *in vitro* to collagens types IV or V attached to Sepharose 2B (table I; collagen preparations were first dialyzed against 0.02 *M* Na_2HPO_4 in an attempt to form fibrils prior to their attachment to Sepharose).

The results of *Freytag et al.* (15) differ substantially from those related above. These authors report that bovine glomerular basement membrane can aggregate platelets. The non-collagenous element of the membrane (obtained after digestion with collagenase) appeared to have some platelet activity, and in this respect their results are in some measure in accord with the findings of *Huang and Benditt* (19) and *Huang et al.* (20), which indicated adhesion to a non-collagenous element. However, the collagenous component (isolated after pepsin digestion) also revealed aggregatory activity. The response of the platelets in this case was characterized by a relatively long lag period which was shortened by preincubation of the test sample at 37 °C prior to addition to platelets. This could perhaps imply the presence in the membrane preparation of trace amounts of interstitial collagen(s), a possibility that was considered previously (20) in regard to earlier reports of platelet-aggregating activity in preparations of kidney basement membranes.

Platelet-Reactivity of the Elastic Fibre

As already related, it is known that platelets can adhere to the subendothelium, a phenomenon that is thought to be important in maintaining normal vascular integrity in the event of loss of endothelium. Attachment of platelets is generally regarded as occurring to the subendothelial basement membrane (7).

However, evidence has been presented that the elastic fibre, and more particularly the microfibrillar element of this structure, also located in the subendothelium at sites where the basement membrane is absent, may be involved in this adhesion (35). We have isolated both elastin and the microfibrillar glycoprotein constituent of the elastic fibre by use of procedures (31, 34) designed to introduce only the minimal alteration in structure compatible with their isolation in a pure state and have examined the platelet reactivity of each. Neither component appeared able to induce platelet aggregation. Furthermore, we detected little, if any, binding of platelets to the microfibrillar element, although some degree of adhesion to elastin was found (table I). Our binding studies are essentially in accord with the findings of *Ordinas et al.* (29) using different methods of isolation of the components of the elastic fibre and a different means of measuring platelet adhesion. We consider our results support the concept that the basement membrane is the important structure as regards platelet adhesion to the subendothelium.

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